Doctoral Thesis

Pax genes as regulators of early Xenopus embryogenesis

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PAX GENES AS REGULATORS OF EARLY XENOPUS EMBRYOGENESIS

A dissertation submitted to the
EIDGENÖSSISCHE TECHNISCHE HOCHSCHULE (ETH) ZÜRICH

for the degree of
Doctor of Natural Sciences

presented by
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1999
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1. Zusammenfassung

Die Entwicklung der Exkretionsorgane im Wirbeltierembryo verläuft in einer räumlich und zeitlich präzise geregelten Abfolge, die durch die Bildung von drei verschiedenen Nieren, der pronephrischen (Vorniere), der mesonephrischen (Urniere) und der metanephrischen Niere (Nachniere) charakterisiert ist. An der Vorniere lassen sich zwei charakteristische Strukturen unterscheiden, die sogenannten Nierentubuli und der Vornierengang (pronephrische oder Wolff 'sche Gang). Der pronephrische Gang nimmt in der Nierenentwicklung eine zentrale Bedeutung ein, da er unter anderem die Ausbildung der mesonephrischen und metanephrischen Niere einleitet. Während die pronephrische Niere der höheren Vertebraten nur eine transiente Struktur darstellt, entwickelt sie sich in Embryonen der Fische und Amphibien zu einem voll funktionsfähigen Organ. Froschembryonen sind daher besonders gut geeignet, um die molekularen Mechanismen der frühen Nierenbildung zu untersuchen.


2. Summary

Development of the excretory system occurs in a series of distinct steps during vertebrate embryogenesis. Three distinct excretory organs, the pronephric, mesonephric, and metanephric kidney, are formed in precise spatial and temporal sequence. The pronephric kidney consists of a tubular system and the pronephric duct which is essential for later steps of vertebrate kidney development. In higher vertebrates, the pronephric kidney is a transient structure. In embryos of fish and amphibia, however, the pronephric excretory system becomes fully functional. Embryos of the frog *Xenopus laevis* represent therefore an ideal model to elucidate the molecular mechanisms underlying early kidney organogenesis.

Pax genes are important developmental control genes as illustrated by mutations in zebrafish, mice and human patients. Among the nine members of the vertebrate Pax gene family, the Pax-2/5/8 genes comprise a separate group which is of particular interest with respect to kidney development. Analysis of Pax-2 deficient mice revealed that Pax-2 might represent a primary regulator of early kidney organogenesis. Furthermore, patients carrying mutations in the Pax-2 gene suffer from renal-coloboma syndrome, an autosomal dominant disease leading to renal insufficiency.

A reverse transcriptase-polymerase chain reaction (RT-PCR) strategy was used to isolate cDNAs encoding all three *Xenopus* Pax-2/5/8 orthologues. Molecular phylogenetic analysis indicates that the amphibian Pax-2/5/8 genes are close relatives of their mammalian counterparts and that all vertebrate Pax-2/5/8 genes are derived from a single ancestral gene. *Xenopus* Pax-2/5/8 genes are expressed in spatially and temporally overlapping patterns during the development of at least seven distinct tissues. Expression of Pax-2 was confined to the nervous system, sensory organs, the visceral arches, and the developing excretory system. In the developing pronephric kidney, Pax-2 expression was initiated at the onset of morphogenesis and detectable in all epithelial structures of the organ. Pax-5 was expressed in the developing midbrain-hindbrain boundary (MHB) and in the otic vesicle, but not in the pronephric kidney. Finally, Pax-8 transcripts were detected in the prospective otic region giving later rise to the otic vesicle and in the developing pronephric kidney. Most strikingly, Pax-8 was identified as the earliest marker of the intermediate mesoderm and thus of the pronephric lineage.

Alternative splicing of Pax gene transcripts is frequently observed, and multiple splice products were also detected for *Xenopus* Pax-2/5/8 transcripts.
The screening of cDNA libraries led to the identification of nine different *Xenopus* Pax-2 cDNAs of which seven encoded novel Pax-2 isoforms. All Pax-2 isoforms retained their DNA-binding domains, but differed significantly in their C-termini. The regulation of Pax-2 splicing was assessed by analyzing different embryonic tissues and whole *Xenopus* embryos. Alternative splicing of Pax-2 transcripts was under temporal regulation during *Xenopus* embryogenesis. Analysis of dissected embryonic heads and pronephric kidneys, as well as animal cap explant cultures could however not provide evidence for tissue-specific splicing.

Several *in vitro* studies have reported differences in the biochemical properties of Pax gene splice variants. Here, the role of Pax-2 isoforms in pronephric kidney organogenesis and MHB development was assessed by injecting *Xenopus* embryos with RNAs encoding single Pax-2 splice variants. Overexpression of Pax-2 proteins led to an enlargement of the expression domains of early and late pronephric marker genes. Pax-2 appears therefore to act at an early step in the molecular cascade controlling kidney development. Ectopic expression of Pax-2 resulted also in enlarged expression domains of MHB markers. Furthermore, ectopic cells expressing MHB marker genes were detected in vicinity to the MHB. The phenotypes obtained in Pax-2 injected embryos suggest a role for Pax-2 in cell fate determination and/or proliferation control during kidney organogenesis and MHB development. Most strikingly, functional differences were observed between different Pax-2 isoforms. These findings suggest that alternative splicing serves to increase the functional diversity of Pax-2 genes during embryogenesis.
3. Introduction

3.1. Principles of embryonic development

Complex, multicellular organisms are a consequence of tightly controlled and coordinated processes during embryogenesis. In the course of embryonic development, undifferentiated cells mature into differentiated cell populations that form tissues and organs with specific functions. Both, embryonic patterning and organogenesis comprise a series of coordinated events that are initiated by inductive signals emitted from one cell population determining the developmental fate of other cells. Once cells become committed to a particular developmental fate, organogenesis and tissue patterning proceed through morphogenesis and progressive differentiation leading to the formation of organs and structures (Gilbert, 1997). The factors directing these complex processes comprise a wide variety of proteins such as secreted factors, membrane-bound receptors, and transcription factors (Kessel and Gruss, 1990).

Although all cells contain the same genetic information, the correct spatial and temporal fate of the different cell populations during embryogenesis is ensured by the expression of an unique subset of the total number of available genes in each cell type. The selected expression of developmental genes requires a reliable, specific, and tight control. Consequently, transcription factors as the most direct regulators of gene transcription play an important role in the development of a multicellular organism (Calkhoven and Ab, 1996). By classical definition, a transcription factor is a protein that binds to DNA in a sequence-specific manner and interacts with other components of the transcriptional machinery to enhance or inhibit transcription of specific target genes (Triezenberg, 1995). Therefore, these nuclear proteins ensure the correct timing and proper localization of gene expression by activating and deactivating corresponding genes. In concordance with their multiple and different tasks in developmental processes and in adult homeostasis, a wide variety of transcription factors does exist. Their ability of specific spatial interaction with DNA is based on various sets of DNA binding motifs which may include homeodomains, paired domains, basic helix-loop-helix domains, basic leucine zipper domains, zinc finger domains, POU domains, winged helix-turn-helix domains, high mobility group (HMG) domains or ETS-domains (Nelson, 1995).
Introduction

3.2. The Pax gene family

Pax genes have been identified as a family of important developmental control genes involved in the formation of various structures and organs in the embryo (Dahl et al., 1997; Mansouri et al., 1996). Pax proteins constitute a small class of transcription factors that harbor a highly conserved DNA-binding motif of 128 amino acids, termed the paired domain, located very close to the amino terminus (Chalepakis et al., 1991; Treisman et al., 1991). Some Pax proteins also contain a complete or truncated paired-type homeodomain, whereby only a complete homeodomain represents a second DNA-binding motif (Adams et al., 1992). In addition, most Pax proteins harbor a conserved stretch of eight amino acids, the octapeptide, of unknown function. Pax proteins diverge mainly in the extreme C-terminus, which constitutes a proline-serine/threonine-rich region and contains sequence motifs mediating transactivation (Chalepakis et al., 1994; Czerny and Busslinger, 1995; Fickenscher et al., 1993).

The paired box motif is found in species of vertebrates and invertebrates indicating its high conservation during evolution. Originally, the paired domain was identified in a class of Drosophila segmentation genes that includes paired, gooseberry-proximal, gooseberry-distal, Pox meso, and Pox neuro (Baumgartner et al., 1987; Bopp et al., 1986). These genes are considered to be involved in the establishment of the Drosophila body plan. In vertebrates, nine Pax genes (designated Pax-1 to Pax-9) have been identified to date (St-Onge et al., 1995). Pax genes have been found in vertebrate species from zebrafish to man. They map on different chromosomal loci and are not organized in clusters (Pilz et al., 1993; Stapleton et al., 1993). On the basis of the assembly of structural motifs, the vertebrate Pax family is classified into four paralogous groups: group I (Pax-1/9), II (Pax-3/7), III (Pax-2/5/8), and IV (Pax-4/6) (Balczarek et al., 1997; Walther et al., 1991) (Fig. 1). Within each group, the genes are characterized by a very high degree of sequence similarity with respect to the paired domains, conservation of genomic organization, and largely overlapping expression patterns. Therefore, the paralogous genes found within each class are likely to be the result of evolutionary duplication and divergence processes (Noll, 1993).
Introduction

<table>
<thead>
<tr>
<th>Pax genes</th>
<th>Basic structure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PD</td>
</tr>
<tr>
<td><strong>Group I</strong></td>
<td></td>
</tr>
<tr>
<td>Pax-1, Pax-9</td>
<td>N</td>
</tr>
<tr>
<td><strong>Group II</strong></td>
<td></td>
</tr>
<tr>
<td>Pax-3, Pax-7</td>
<td>N</td>
</tr>
<tr>
<td><strong>Group III</strong></td>
<td></td>
</tr>
<tr>
<td>Pax-2, Pax-5, Pax-8</td>
<td>N</td>
</tr>
<tr>
<td><strong>Group IV</strong></td>
<td></td>
</tr>
<tr>
<td>Pax-4, Pax-6</td>
<td>N</td>
</tr>
</tbody>
</table>

Fig. 1. Structure of vertebrate Pax proteins. All Pax proteins contain a paired domain (PD), a DNA binding domain of 128 amino acids, located at their amino-terminal ends. Distinct subgroups of proteins are defined by the presence or absence of a paired-type homeobox (HD) and an octapeptide motif (OP). In addition to the paired domain, Pax-3, Pax-4, Pax-6, and Pax-7 have a second DNA binding domain, the paired-type homeodomain (HD). This homeodomain is truncated in Pax-2, Pax-5 and Pax-8, and is totally absent in Pax-1 and Pax-9. Furthermore, all Pax proteins, except Pax-4 and Pax-6, carry an octapeptide motif located between the paired domain and the homeobox. Figure adapted from Dahl et al. (1997).

3.3. Expression of Pax genes during vertebrate development

The expression of Pax genes during embryogenesis has been studied most comprehensively in the mouse. Each member of the Pax gene family shows spatially and temporally restricted expression patterns during embryogenesis.

All Pax genes, except Pax-1 and Pax-9, are expressed in various restricted territories of the developing nervous system implicating a crucial role in the regionalization of the neural tube and the brain (Chalepakis et al., 1993). Pax-6 is found in the telencephalon (Walther and Gruss, 1991); Pax-3 and Pax-7 in the mesencephalon (Goulding et al., 1991; Jostes et al., 1990); and Pax-2, Pax-5, and Pax-8 at the midbrain-hindbrain boundary (Asano and Gruss, 1992; Nornes et al., 1990; Plachov et al., 1990). In the spinal cord, Pax genes display restricted expression patterns along the dorsoventral axis (Chalepakis et al.,
In the developing eye, Pax-2 is exclusively expressed in the optic stalk, while Pax-6 expression is localized in the eye cup (reviewed in Macdonald and Wilson, 1996).

Besides expression in the developing nervous system, several Pax genes are also expressed during development of various organs. During somitogenesis, Pax-1, -3, -7, and -9 are expressed in restricted patterns within the paraxial mesoderm. After somite formation, Pax-1 and Pax-9 expression becomes confined to the sclerotome, whereas expression of Pax-3 and Pax-7 is associated with the dermomyotome (St-Onge et al., 1995). Pax-1 is also expressed in the thymus (Wallin et al., 1996); Pax-4 and Pax-6 are found in the pancreas (Sosa-Pineda et al., 1997; Turque et al., 1994); Pax-5 is detected in early B-lymphopoiesis (Adams et al., 1992); and Pax-8 is associated with the thyroid gland (Plachov et al., 1990). Finally, Pax-2 and Pax-8 expression is observed in the developing kidney (Dressler et al., 1990; Plachov et al., 1990).

3.4. Alternative splicing of Pax genes

The complexity of Pax protein distribution and function during development is enhanced by alternative splicing. Alternative splicing is an important mechanism generating protein diversity from a single gene by selectively excising different exons encoded by that gene during RNA processing (Smith et al., 1989). Transcripts that either contain or lack particular exons may have entirely different biochemical properties at the protein level (Foulkes and Sassone-Corsi, 1992). Particularly, transcription factors are highly modular in the organization of sequence elements required for DNA binding, dimerization, ligand binding, subcellular localization, and transcriptional activation (reviewed by Mitchell and Tjian, 1989). Interestingly, isoforms with opposite (activating or repressing) functions are often generated from the same gene by modifications in the domain responsible for the activation of transcription of target genes. Transcription factor isoforms lacking the functional activator domain, while still performing an efficient and specific recognition of target DNA motifs, act as negative regulators of transcription by competing for binding to the same DNA site with the corresponding activator domain containing isoform (López, 1995).

Alternative splicing is a common phenomenon for Pax genes. Multiple splice products have been reported for nearly all members of the Pax gene family. In vitro studies revealed that alternative splicing can modulate DNA binding affinity and specificity as well as transactivation activity. For example, insertion of an additional exon in the paired domain of Pax-6 results in
modification of the DNA binding specificity suggesting that the two isoforms regulate different sets of target genes (Epstein et al., 1994b). Furthermore, alternate use of consensus 3' splice sites lead to a deletion of three nucleotides in the paired domain of Pax-3 and Pax-7 proteins generating isoforms with increased affinity to target DNA sequences (Vogan et al., 1996). For the Pax-8, at least seven splice variants have been identified that do not differ in DNA binding activity but demonstrate distinct transactivation properties. Moreover, changes in the relative abundance of different splice variants during embryogenesis indicate that alternative splicing may be developmentally regulated (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995).

3.5. Functions of Pax genes in embryonic development

Based on their embryonic expression patterns, Pax genes may exert multiple functions during embryogenesis. The present understanding of Pax gene function is based mainly on in vivo studies. Analysis of ectopic expression experiments in Drosophila has identified Pax-6 as a master regulator of eye development (Halder et al., 1995). Furthermore, naturally occurring and genetically engineered mutations in Drosophila, zebrafish, mouse, and man have shed light on the requirement of Pax gene function for embryonic development. These findings will be discussed below in greater detail. Many efforts have also been directed towards the identification of target genes to elucidate the role of Pax genes in the hierarchical network of gene regulation during embryogenesis (Adams et al., 1992; Epstein et al., 1994a; Epstein et al., 1996; Song et al., 1996; Zannini et al., 1992). Finally, in vitro data indicate that an important function of Pax genes during organogenesis could be the initiation of cell proliferation (Maulbecker and Gruss, 1993; Stuart et al., 1995).

3.6. Mutations in Pax genes

Clinical and molecular analysis of human diseases and mouse mutants caused by Pax gene mutations suggests that vertebrate Pax genes are key regulators during embryonic development of the kidney (Pax-2), the thyroid gland (Pax-8), the pancreas (Pax-4, -6), the teeth (Pax-9), the eye (Pax-2, -6), the ear (Pax-2), the nose (Pax-6, -7), the limb muscle (Pax-3), the vertebral column (Pax-1), the brain (Pax-2, -5), the derivatives of the third and fourth pharyngeal pouches (Pax-9), and the neural crest derivatives (Pax-3, -7). Moreover, a remarkable characteristic of mutations at Pax gene loci is that the loss of one allele is sufficient to exhibit a dominant phenotype, also known as
haploinsufficiency. Although haploinsufficiency itself is a rare case among vertebrate transcription factors (Engelkamp and van Heyningen, 1996), Pax proteins seem to exhibit a remarkable degree of dosage sensitivity. Therefore, a correct dosage of these Pax proteins appears to be essential to ensure normal development.

3.6.1. Naturally occurring Pax mutants

The importance of Pax genes for embryonic development is emphasized by the fact that naturally occurring mutations in Pax genes have been associated with congenital abnormalities in mouse and man (Dahl et al., 1997; Hill and Hanson, 1992; Read, 1995; summarized in Table 1). In most cases, an obvious correlation between the expression patterns of Pax genes and the manifestation of abnormalities in the corresponding mutant phenotypes can be seen.

Table 1. Diseases in mouse and man associated with Pax gene mutations

<table>
<thead>
<tr>
<th>Pax genes</th>
<th>Mouse mutant (natural)</th>
<th>Human syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax-1</td>
<td>undulated (un/un&lt;sup&gt;ex&lt;/sup&gt;/Un&lt;sup&gt;s&lt;/sup&gt;)</td>
<td>Spina bifida (?)</td>
</tr>
<tr>
<td>Pax-3</td>
<td>Splotch</td>
<td>Waardenburg syndrome I and III</td>
</tr>
<tr>
<td>Pax-2</td>
<td>Pax-2&lt;sup&gt;1&lt;/sup&gt;Neu</td>
<td>Renal coloboma syndrome</td>
</tr>
<tr>
<td>Pax-8</td>
<td></td>
<td>Congenital hypothyroidism (CH)</td>
</tr>
<tr>
<td>Pax-6</td>
<td>Small eye (Sey)</td>
<td>Aniridia; Peter's anomaly</td>
</tr>
</tbody>
</table>

(adapted from Dahl, 1997)

In mouse, the Pax-1 gene has been found to be mutated in three undulated alleles causing malformations of the vertebral column (Dahl et al., 1997; Wilm et al., 1998). The phenotypes range from mild skeletal abnormalities to severe skeletal malformations in the different heterozygous conditions. In undulated (un), an amino acid substitution in the paired domain alters the DNA binding affinity and specificity of the mutated protein (Balling et al., 1988; Chalepakis et al., 1991). The undulated extensive (un<sup>ex</sup>) allele harbours a deletion that includes the last exon of the Pax-1 gene (Dietrich and Gruss, 1995). In the Undulated short-tail (Un<sup>s</sup>) allele, the entire Pax-1 locus is absent (Wallin et al., 1996). In humans, it is not yet known whether mutations in
the Pax-1 gene are also associated with congenital diseases. Recent reports indicate, however, that mutations in the Pax-1 gene could contribute to the pathogenesis of spina bifida in mouse and human (Helwig et al., 1995; Hol et al., 1996).

A deletion in the mouse Pax-3 gene causes the Splotch phenotype (Epstein et al., 1991). Homozygous Splotch mice show severe defects in closure of the neural tube (exencephaly and spina bifida), impaired limb muscle development and malformations in neural crest derivatives. Mutations in the human Pax-3 gene were found in patients with Waardenburg syndrome (Baldwin et al., 1992; Tassabehji et al., 1992; Tassabehji et al., 1993; Morell et al., 1992). This clinically and genetically heterogenous disorder is characterized by numerous defects in neural crest-derived tissues.

Eye defects found in species ranging from Drosophila to humans as a consequence of mutations in the Pax-6 gene illustrate the evolutionary conservation of Pax gene function in embryogenesis (Halder et al., 1995; Hanson and van Heyningen, 1995). In Drosophila, mutations in the gene homologous to Pax-6 are responsible for the eyeless phenotype (Quiring et al., 1994). In the mouse, homozygous Small eye (Sey) mutants completely lack eyes and nasal cavities, whereas heterozygous animals show a reduction in eye size (Hill et al., 1991). In the human syndrome aniridia, Pax-6 mutations range from single base pair mutations to large deletions (Hill et al., 1991; Ton et al., 1991). The patients suffer from complete or partial absence of the iris and defects in cornea, lens, retina, and optic nerve. Additionally, mutations at the Pax-6 locus in man have been described for Peter’s anomaly (Hanson et al., 1994).

Recently, mutations have been also identified in genes of the Pax-2/5/8 class. Renal-coloboma syndrome has been correlated with heterozygosity for natural Pax-2 gene mutations in human families (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). In mouse, a spontaneous mutation of the Pax-2 gene (Pax-2Neu1) has been reported that cause a similar phenotype as described for patients with renal-coloboma syndrome (Favor et al., 1996). In human newborns, congenital hypothyroidism due to thyroid dysgenesis is a common disease. Mutations in the Pax-8 gene have been implicated in the pathogenesis of thyroid dysgenesis. Recently, patients with heterozygous Pax-8 mutations were described, which suffer from impaired thyroid gland function due to thyroid hypoplasia. Three separate Pax-8 mutations were identified which cause severe reduction in DNA binding and transactivation activity (Macchia et al., 1998).
3.6.2. Genetically engineered Pax mutants

Over the last few years, several new mouse strains have been generated by gene targeting methods to create defined Pax null alleles (Table 2).

Table 2. Phenotypes of homozygous mice with targeted deletions of Pax genes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pax-1</td>
<td>Viable and fertile; skeletal abnormalities along the vertebral column, in the sternum and the scapula</td>
<td>Wilm et al. (1998)</td>
</tr>
<tr>
<td>Pax-9</td>
<td>Perinatal lethal; lack of derivatives of the third and fourth pharyngeal pouches (thymus, parathyroid gland, ultimobranchial body); absence of teeth; abnormalities of head and visceral skeleton; supernumerary digits; lack of the flexor of the hindlimb toes</td>
<td>Peters et al. (1998)</td>
</tr>
<tr>
<td>Pax-7</td>
<td>Postnatal lethal; dysgenesis of cephalic neural crest derivatives (malformations in facial structures involving the maxilla and nose)</td>
<td>Mansouri et al. (1996)</td>
</tr>
<tr>
<td>Pax-2</td>
<td>Embryonic lethal; lack of kidneys, ureters and genital tracts; eye defects (agenesis of the optic chiasma, optic nerve coloboma); inner ear defects (agenesis of the cochlea and the spiral ganglion); mouse strain dependent exencephaly (30-100%)</td>
<td>Torres et al. (1995); Torres et al. (1996); Schwarz et al. (1997)</td>
</tr>
<tr>
<td>Pax-5</td>
<td>Postnatal lethal; 5% viable and fertile; defect in B-cell development; mild defect of the posterior midbrain (reduction of the inferior colliculus) and altered foliation of the anterior cerebellum</td>
<td>Urbánek et al. (1994)</td>
</tr>
<tr>
<td>Pax-8</td>
<td>Postnatal lethal; thyroid gland defects (lack of thyroxine-producing follicular cells)</td>
<td>Mansouri et al. (1998)</td>
</tr>
<tr>
<td>Pax-4</td>
<td>Postnatal lethal; pancreas defects (lack of mature insulin- and somatostatin-producing cells)</td>
<td>Sosa-Pineda et al. (1997)</td>
</tr>
<tr>
<td>Pax-6</td>
<td>Perinatal lethal; lack of eyes and olfactory bulbs; pancreas defects (lack of mature glucagon-producing cells)</td>
<td>St-Onge et al. (1997)</td>
</tr>
</tbody>
</table>
3.7. Pax genes in the development of the midbrain-hindbrain boundary

The midbrain-hindbrain boundary (MHB, mesencephalic-metencephalic boundary, or isthmus) represents an important signaling center that controls the establishment of specific brain structures during embryonic development (Joyner, 1996). The MHB has been identified as a major site of Pax gene expression during neural development. Expression of Pax-2, Pax-5, and Pax-8 overlaps spatially and temporally at the MHB with Pax-2 being the first gene and Pax-8 being the latest to be expressed (Asano and Gruss, 1992; Krauss et al., 1991; Nornes et al., 1990; Pfeffer et al., 1998).

3.7.1. Development of the vertebrate central nervous system

In vertebrate embryos, the central nervous system forms along the dorsal midline of the body axis. Its formation is the consequence of a series of inductive interactions and is initiated during gastrulation, when dorsal mesodermal cells moves into contact with the overlying ectoderm. In this process, known as neural induction, mesodermal cells influence ectodermal cells to adopt a neural fate. Subsequently, the neural ectoderm folds up to form the neural tube and regionalizes along its anteroposterior axis in response to inductive signals emanating from the dorsal mesoderm or produced in restricted region of the developing neural epithelium (Kelly and Melton, 1995; Lumsden and Krumlauf, 1996). The appearance of three swellings (vesicles), named prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain), in the rostral neural tube is the first morphological indication of a segmented organization of the developing brain. The prosencephalon is then divided into the telencephalon and the diencephalon, and later into smaller prosomers (Rubenstein et al., 1994). The rhombencephalon is further divided into the metencephalon and myelencephalon, with the latter being further segmented into smaller rhombomeres. In contrast, the mesencephalon and metencephalon do not appear to be further segmented (Joyner, 1996).

Chick-quail homotypic and heterotypic transplantation studies have given insights in cell fate and cell commitment of distinct brain regions (Wassef and Joyner, 1997). They have revealed that the cerebellum is derived from both the caudal mesencephalon and the metencephalon, whereas the tegmentum and colliculi (tectum) are derived from more rostral regions of the mesencephalon. Interestingly, the MHB has been identified as an organizer center controlling the development of the surrounding neuroepithelium. Tissue grafts from the MHB
Introduction

region were shown to induce an ectopic midbrain or cerebellum when transplanted into the chicken forebrain or hindbrain, respectively (Bally-Cuif and Wassef, 1995).

3.7.2. Signaling events in the development of the MHB

A large effort has been directed towards identification of regulatory genes to gain insight into the underlying molecular events that comprise the complex set of intrinsic and extrinsic instructions that pattern the brain. Over the past decade, a number of molecules with supposed regulatory and signaling functions required for the proper development of the MHB territory has been identified (reviewed in Joyner, 1996; Wassef and Joyner, 1997). Members of the Engrailed (En), Pax, Wnt and FGF gene families are expressed in spatially restricted domains during early development of the MHB, most of them being homologues of Drosophila segmentation genes. En-1, En-2, Wnt-1, FGF-8, Pax-2, Pax-5 and Pax-8 are transiently expressed in overlapping domains surrounding the midbrain-hindbrain junction (Chalepakis et al., 1993; Davis and Joyner, 1988; Rowitch and McMahon, 1995). The present understanding of their function in MHB development is mainly based on the analysis of genetically engineered mouse mutants. Targeted mutations of these genes revealed that loss-of-function causes similar abnormalities of variable strength in early brain development. These genes appear to be involved in early specifications, proliferation, and/or survival of the ventricular layer cells that give rise to the MHB derivatives. Variations in phenotype severity might be a consequence of differences in spatial and temporal gene expression and redundant gene function among gene family members. Furthermore, gene disruption studies in zebrafish and gain-of-function experiments in chicken have contributed to elucidate molecular mechanisms underlying MHB development.

3.7.2.1. Consequences of Pax-2/5/8 gene disruptions for MHB development in the mouse

Inactivation of murine Pax-5 leads only to a mildly abnormal development of the anterior midbrain and the posterior cerebellum (Urbánek et al., 1994). Pax-2 homozygous mutant mice also develop essentially normal MHB derivatives (Schwarz et al., 1997). However, analysis of double Pax-2/Pax-5 mutants reveals a more severe phenotype than that obtained in single mutants suggesting functional redundancy between the two gene family members (Schwarz et al., 1997). Analysis of Pax-8 homozygous mutants does not reveal
any defects in MHB derivatives implicating compensatory function of Pax-2 and/or Pax-5 in the mutant animals (Mansouri et al., 1998).

3.7.2.2. Consequences of Pax-2.1 gene disruption for MHB development in the zebrafish

In contrast to the homozygous Pax-2 mouse mutants, chemically induced inactivation of the zebrafish Pax-2.1 gene shows a severe phenotype in zebrafish embryos. The noi (no isthmus) mutant is characterized by the loss of the midbrain and cerebellum (Brand et al., 1996; Lun and Brand, 1998). A similar phenotype with malformations at the MHB can be induced by injection of antibodies raised against the Pax-2.1 protein (Krauss et al., 1992). During zebrafish embryogenesis, Pax-2.1 expression at the MHB precedes that of Pax-5 and Pax-8. Noteworthy, Pax-5 and Pax-8 expression at the MHB strictly depends on Pax-2.1 function. Therefore, inactivation of Pax-2.1 can be considered as a functionally equivalent to triple inactivation of Pax-2/5/8 genes. These findings explain the phenotypic differences between mice and zebrafish Pax-2.1 mutants (Brand et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998).

3.7.2.3. Consequences of altering gene dosage of other factors for MHB development

Both, Wnt-1 and En-1 deficient mice have deletions of the cerebellum and the midbrain with Wnt-1 deficiency resulting in a more severe phenotype (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). En-2 mutants show a reduction in size of the cerebellum with an abnormal folding pattern and alterations of the colliculi (Millen et al., 1994). En-1/En-2 double mutants have a more severe deletion phenotype than the single mutants consistent with compensatory function between the two En proteins (Hanks et al., 1995). FGF-8 gene inactivation in mice causes cerebellar and midbrain defects (Meyers et al., 1998). Conversely, beads soaked in recombinant FGF-8 protein and implanted in the caudal forebrain can mimic the effect of MHB grafts by inducing ectopic midbrain structures in chick embryos (Crossley et al., 1996).
3.7.2.4. Conclusions

Analysis of mouse and zebrafish mutants has enabled the identification of many transcription factors and secreted molecules that function in the development of the MHB territory. Nevertheless, the hierarchies of interactions of these factors and the molecular mechanisms which allow the establishment, the further subdivision, and full development of the complement of midbrain and cerebellar structures are still poorly understood. One reason for the difficulty to dissect the complexity of the developmental program in mutant animals is the functional redundancy observed for several components. Therefore, approaches with multiple experimental strategies and model systems will shed more light on the unresolved questions.

3.8. Pax genes in kidney organogenesis

3.8.1. Development of the vertebrate excretory system

During vertebrate embryogenesis, the excretory system develops from the intermediate mesoderm by successive formation of three distinct kidneys along the rostrocaudal axis of the embryo, namely the pronephric, the mesonephric, and the metanephric kidney (Saxén, 1987) (Fig. 2).

Vertebrate kidney organogenesis is initiated by induction of the pronephric kidney. The pronephric kidney consisting of the pronephros and the pronephric duct (also known as nephric or Wolffian duct) is established as the earliest kidney and represents the simplest form of an excretory organ in vertebrates. In embryos of fish and amphibians, the pronephric kidney develops to a fully functional organ, while it remains a transient structure in higher vertebrates. The pronephric duct constitutes the central component of the excretory system throughout development (Herzlinger, 1995). It is the drainage channel of the functional pronephric and mesonephric kidney, it gives rise to the ureteric bud of the metanephros and ultimately contributes to the male genital system as the ductus deferens. Moreover, the epithelium of the pronephric duct and its derivatives induce the morphogenesis and differentiation of the mesonephric and metanephric mesenchymal nephric cord into tubular structures. In fish and amphibians, the mesonephric kidney represents the excretory organ of the adult animal. In reptiles, birds and mammals, however, the mesonephric kidney is succeed by the metanephric kidney. Development of the metanephric kidney is initiated when the ureteric bud induces nephrogenic mesenchymal cells to condense and create an epithelial aggregate that will give rise to the renal nephron. As an example of reciprocal induction, the condensed mesenchyme
will induce the ureteric bud to branch repeatedly to form the renal collecting ducts and the ureter (Grobstein, 1955).

**Fig. 2.** Overall scheme of vertebrate kidney development. (A) The pronephros and the nephric duct form from the nephrogenic cord. (B) The pronephros degenerates, whereas the elongating nephric duct induces by interacting with the nephrogenic cord the formation of the mesonephros and remains as the drainage channel of the functional mesonephros. (C) Development of the metanephric kidney is initiated by the ureteric bud, an outgrowth of the caudal aspect of the nephric duct that induces the nephrogenic mesenchyme to condense. The mesonephros also degenerates but is still seen during early stages of the metanephros development. Figure taken from Burns (1955).

### 3.8.2. The pronephric kidney as a model system to study kidney organogenesis

Based on histology, morphology and classical experimental embryology (Fox, 1963; Vize et al., 1995; Vize et al., 1997), it appears that the formation of the pronephric kidney requires many of the same mechanisms necessary for
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later steps of kidney organogenesis such as inductive interactions, mesenchyme-to-epithelial transitions, cell migration and patterning formation along a tubular system. All three kidneys have a similar functional organization and differ mostly in the spatial assembly within the organism and the number and types of nephrons (Saxén, 1987; Vize et al., 1995; Vize et al., 1997). While the metanephric kidney of humans contains up to 1 million nephrons, the pronephric kidney of amphibians is typically composed of 2-3 nephrons. The nephron is the structural and functional unit of the vertebrate excretory system ensuring excretion and osmoregulation. Each nephron consists of a glomerulus and a segmented tubular system. In the pronephros, however, a single vascularized glomus replaces individual glomeruli. The glomus filters waste from the blood into the nephrocoel or coelom. Associated coiled pronephric tubules collect the filtrate through ciliated funnels (nephrostomes) and return resorbed materials to the blood. The filtrate remaining after completion of tubular resorption is disposed as urine via the pronephric duct (Fig. 3). Bilateral removal of the pronephroi in embryos of lower vertebrates results in edema and death, whereas unilateral extirpation leads to compensatory hypertrophy of the remaining pronephros (Fales, 1935; Holtfreter, 1944; Howland, 1927).

For the analysis of metanephric kidney development, the use of an in vitro organ culture system and genetic evidence provided by transgenic animal models have helped to define essential components of an emerging network of signaling pathways that regulates its development (Ekblom, 1996; Herzlinger, 1995; Lechner and Dressier, 1997; Vainio and Müller, 1997). Interestingly, genes that have been identified to be critical in normal metanephric kidney development are also expressed in the pronephros or in the early mesoderm (Davies and Brändli, 1996; Vize et al., 1997) indicating that these genes play similar roles in the development of all three kidneys. Taken together, the pronephric kidney can serve as an simplified organ model to study the molecular and cellular basis of vertebrate kidney formation.

3.8.3. Expression of Pax genes in the developing excretory system

Expression of Pax-2 and Pax-8 is associated with all three stages of kidney organogenesis. In the pronephric kidney, the presence of Pax-2 and Pax-8 has been reported in human, mouse and zebrafish (Dressler and Douglass, 1992; Krauss et al., 1991; Pfeffer et al., 1998; Terzic et al., 1998). During mesonephric development, Pax-2 transcripts are observed in the mesonephric tubules and in the Wolffian duct. Once the mesonephros
degnerates, Pax-2 expression is maintained in the ureteric bud, an outgrowth of the caudal aspect of the Wolffian duct, and is activated in the condensing metanephric mesenchyme and its early epithelial derivatives. Expression is rapidly downregulated as terminal differentiation of the renal tubules proceeds (Dressler and Douglass, 1992; Eccles et al., 1992). In contrast, Pax-8 expression is restricted to more differentiated stages in mesonephric and metanephric development. During metanephric kidney organogenesis, Pax-8 expression first appears in renal vesicles, comma- and S-shaped bodies, but is absent in the ureteric bud and condensing mesenchyme (Poleev et al., 1992).

**Fig. 3.** Organization of the pronephric kidney. Wastes filtered from the glomus into the coelom are collected by the tubules via the nephrostomes. Resorbed materials are returned to the blood stream via the pronephric sinus (blue) which is derived from the posterior cardinal vein and surrounds the tubes. Filtrate remaining after tubular resorption is disposed as urine via the pronephric duct. Figure taken from Vize et al. (1997).
3.8.4. Functions of Pax genes in kidney development

Strict control of Pax-2 expression levels appears to be important for normal metanephric development. During metanephric kidney organogenesis, Pax-2 is rapidly downregulated as terminal differentiation of the renal epithelium proceeds (Dressler et al., 1990; Dressler and Douglass, 1992; Eccles et al., 1992). Failure to repress Pax-2 in transgenic mice results in severe kidney abnormalities presumably by interfering with terminal differentiation of renal epithelial cells (Dressler et al., 1993). Downregulation of Pax-2 is believed to be partially mediated by Wilms' tumor suppressor (WT-1). A significant increase in WT-1 protein levels coincides precisely with downregulation of Pax-2 expression in precursor cells of the glomerular epithelium. Indeed, WT-1 directly inhibits Pax-2 transcription in vitro (Ryan et al., 1995). Furthermore, both Pax-2 and Pax-8 are expressed at elevated level in undifferentiated mesenchymal cells of Wilms' tumor (Dressler and Douglass, 1992; Eccles et al., 1995), a pediatric tumor of the kidney that is associated with mutations in WT-1 (reviewed by Coppes, 1995). Vice versa, the WT-1 promoter is actively stimulated when cotransfected with Pax-2 in vitro. Deletion mutagenesis of the WT-1 promoter identified potential binding sites for Pax-2 (Dehbi et al., 1996; McConnell et al., 1997). Overexpression of Pax-2 in human kidney cells (HEK293) leads to a 2-fold increase of WT-1 expression (Torban and Goodyer, 1998). Together, these findings suggest a regulatory mechanism by which WT-1 and Pax-2 control each other's expression.

In vitro experiments using Pax-2 antisense oligonucleotides in kidney organ cultures have indicated that Pax-2 is required for mesenchyme-to-epithelium conversion in metanephric tubule formation (Rothenpieler and Dressler, 1993). With significantly reduced Pax-2 protein levels, mesenchymal kidney cells fail to aggregate and do not undergo sequential morphological changes characteristic for epithelial cell formation. Overexpression studies of Pax-2 in human embryonic kidney cells (HEK293) suggest that Pax-2 promotes the mesenchyme-to-epithelium transition by modifying the expression of genes critical to the epithelial phenotype. Elevated levels of Pax-2 protein lead to upregulation of E-cadherin and suppression of vimentin expression (Torban and Goodyer, 1998). Taken together, the in vitro studies have enabled the identification of possible molecular interactions and the elucidation of some of the functional aspects of Pax-2 during metanephric development.
3.8.4.1. Consequences of Pax-2 mutations

Additional insights into the role of Pax genes in kidney development have emerged from genetic analysis in zebrafish, mice and humans. To date, several human and three mouse Pax-2 mutations have been described. Heterozygous carriers of these mutations express eye and kidney abnormalities with a variability in expressivity. In addition, zebrafish with mutations in the Pax-2.1 gene have been recently reported.

Human Pax-2 mutations cause renal-coloboma syndrome

The renal-coloboma syndrome (optic nerve coloboma-renal disease, papillo-renal syndrome) is a rare disorder characterized by autosomal dominant inheritance of optic, auditory, and renal defects (Weaver et al., 1988). Ocular manifestations in this syndrome are variable anomalies of retinal and optic disk dysplasia. Anomalies of the optic nerve and thinning of the retinal epithelium results in loss of visual acuity and defective visual fields. Patients suffer simultaneously from chronic renal failure, chronic glomerulonephritis and/or renal hypoplasia. Occasionally, the renal-coloboma syndrome is associated with hearing defects.

Recently, renal-coloboma syndrome has been correlated with heterozygosity for natural Pax-2 gene mutations in unrelated human families (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). Over the last three years, analysis of patients with renal-coloboma syndrome has led to the identification of at least six mutations (for review, see Online Mendelian Inheritance in Man (OMIM) homepage; http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/). Both, truncating mutations as well as missense mutations in the human Pax-2 gene have been identified (summarized in Fig. 4).

Two independent insertions of a single base pair within the paired domain (exon 2) lead to frameshift mutations resulting in severely truncated proteins (Sanyanusin et al., 1995a; Schimmenti et al., 1997). Another frame shift mutation in the paired domain is caused by a deletion of 22 bp in exon 2 (Schimmenti et al., 1997). Furthermore, individuals of other affected families carry a deletion of a single nucleotide in the octapeptide (exon 5) that results in a truncated protein which lacks the partial homeodomain and the entire C-terminus (Sanyanusin et al., 1995b; Schimmenti et al., 1995). More recently, a heterozygous missense mutation and a heterozygous hexanucleotide duplication in the paired domain (exon 3) of the Pax-2 gene of unrelated
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families have been described that do not lead to truncated proteins (Devriendt et al., 1998).

Fig. 4. Depiction of the location of the mutations within the human Pax-2 protein causing renal-colioboma syndrome. The structure of the Pax-2 protein is shown schematically with the corresponding domain organization (PD, paired domain; OP, octapeptide motif; HD, homeodomain). Exons numbered from 1 to 10 according to Pfeffer et al. (1998) are present in all Pax-2/5/8 members, whereas the suffix '1.' refers to less well conserved, non-canonical exons. Above, positions of the missense mutations are indicated that preserve the original reading frame of the Pax-2 protein. Below, positions of mutations are indicated that introduce frame shifts resulting in truncated Pax-2 proteins.

In principle, these mutant Pax-2 proteins can lead to a disruption of transcription factor complexes, or to a competitive inhibition of DNA binding of the normal Pax-2 protein. The frame shift mutations in the paired domain of Pax-2 causing severely truncated proteins with a lack of DNA binding and transactivation activities can be regarded as loss-of-function mutations. The missense mutations in the paired domain are expected to result in abnormal folding of the Pax-2 protein that might results in reduction or loss of DNA binding and transactivation activity. The frame shift mutation in the octapeptide motif leads to a truncated protein with an intact paired domain that would still be able to bind DNA but obviously lacks the ability to transactivate the expression of target genes. In summary, the reduced dosage of functional Pax-2 protein appears to be at the basis of the eye and renal malformations.
Pax-2 mutations and vesicoureteric reflux

Vesicoureteric reflux (VUR) is a common childhood condition characterized by regurgitation of urine from the bladder to the kidney (Bailey, 1979) causing severe kidney damage (reflux nephropathy) and even leading to end stage renal failure in a significantly high percentage of patients (Bailey et al., 1984). VUR is a result of a congenital defect of the length, diameter, musculature or innervation of the submucosal segment of the ureter (Stephens and Lenaghan, 1962). A developmental abnormality of the caudal portion of the ureteric bud is believed to be the primary defect (Stephens, 1976). VUR is frequently clustered in families (Heale et al., 1979) suggesting its genetic origin. Normally, hereditary VUR is not associated with other anomalies (primary familial VUR), but in rare instances VUR occurs in association with other diseases, such as the renal-coloboma syndrome, which is caused by a Pax-2 gene mutation (Sanyanusin et al., 1995b). An extensive survey of patients suffering from VUR by single stranded conformational polymorphism analysis revealed no alteration in the Pax-2 gene. In summary, Pax-2 mutations are not considered as a major cause of primary familial VUR (Choi et al., 1998; Eccles et al., 1996).

Murine Pax-2 mutants

Three mouse strains with mutations in the Pax-2 locus have been described. The Krd (kidney and retinal defects) strain carries a large transgene induced chromosomal deletion (~7 cM) including the Pax-2 locus. They develop retinal abnormalities and a high incidence of kidney defects (hypoplasia) (Keller et al., 1994). Although these phenotypic features observed in the Krd mouse might be attributed also to the loss of other genes, the effect is dominant and resembles that observed in human patients heterozygous for a Pax-2 gene mutation.

Torres et al. (1995) generated a Pax-2 knock out mutation in the mouse. Heterozygous mutants frequently showed reduction in kidney size. The analysis of homozygous mouse mutants indicates that Pax-2 controls multiple steps during urogenital development as demonstrated by the absence of mesonephric and metanephric kidneys, ureters and genital tracts (Torres et al., 1995). The phenotype has led to the proposal that Pax-2 might be a primary regulator for these developmental processes. Similar to humans, Pax-2 mutant mice show eye (optic nerve coloboma) and inner ear defects (Torres et al., 1996).
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Favor et al. (1996) identified a spontaneous murine frameshift mutation (Pax2\(^{1\text{Neu}}\)) with a 1-bp insertion in the Pax-2 gene identical to that found in humans (Sanyanusin et al., 1995a). Heterozygous mutant embryos display a similar phenotype as described for human families with renal-coloboma syndrome by exhibiting defects in the kidney, the optic nerve, and the retinal layer of the eye. The homozygous animals reveal similar phenotypes as observed in the knock out mutants.

**Zebrafish Pax-2 mutants**

In zebrafish, two Pax-2 like genes called Pax-2.1 and Pax-2.2, respectively, have been identified (Krauss et al., 1991; Pfeffer et al., 1998). Similar to mouse Pax-2, zebrafish Pax-2.1 is expressed during kidney organogenesis as well as in the developing MHB, spinal cord, eye, and ear. Pax-2.2 differs from Pax-2.1 by the absence of expression in the nephric system and by a delayed onset of transcription in other tissues with Pax-2.1 expression. The chemically induced no isthmus (noi) mutations is known to result from mutations in the Pax-2.1 gene (Brand et al., 1996; Lun and Brand, 1998). During embryogenesis, mutant embryos form rudimentary ducts that fail to elongate and subsequently degenerate (Brand et al., 1996).

3.8.4.2. Consequences of Pax-8 mutations

Patients heterozygous for mutations in the Pax-8 gene display severe hypothyroidism, characterized by a dramatic reduction of the thyroid gland (Macchia et al., 1998). Inactivation of murine Pax-8 leads to thyroid dysgenesis due to the lack of follicular cells. However, no defects have been observed in other structures expressing Pax-8 (Mansouri et al., 1998). Although Pax-8 is expressed both during early and late vertebrate kidney development, mutations in the Pax-8 gene of mice and man affect obviously only the organogenesis of the thyroid gland and do not lead to kidney defects probably due to partially redundant function provided by Pax-2 during kidney organogenesis.

3.8.4.3. Conclusions

Overall, animals deficient in Pax genes are powerful tools to study Pax gene function in kidney organogenesis. Determination of the role of Pax genes in kidney development is however hampered by restricted experimental access in mouse and man. Furthermore, the interpretation of Pax gene function
analysis becomes complex due to functional redundancy and alternative splicing. Complementary animal models like *Xenopus laevis* and zebrafish may therefore provide novel insights concerning the role of Pax genes in kidney organogenesis.

### 3.9. *Xenopus laevis* as a novel model for the analysis of kidney organogenesis

Given its transient nature in higher vertebrates, pronephric kidney organogenesis is ideally studied in lower vertebrates. In fact, the pronephric kidney was first described in toad and frog embryos at the beginning of the nineteenth century (Müller, 1829). In many areas of developmental biology, the frog *Xenopus laevis* has become the experimental organism of choice over the last four decades. Despite the lack of classical genetics, the major advantage of the *Xenopus* system is the possibility of generating thousands of embryos and the ease with which they can be manipulated by microdissection. In addition, simple microinjection techniques and novel transgenic methods (Kroll and Amaya, 1996) provide powerful tools to elucidate gene function.

In *Xenopus* embryos, the development of the pronephric kidney into a functional organ occurs very rapidly and is completed within 2-3 days after fertilization (Nieuwkoop and Faber, 1994). The availability of a suitable *in vitro* organ culture system was essential in order to define the inductive interaction occurring during metanephric development (Saxén, 1987). Interestingly, the induction and formation of pronephric tubules can be reproduced *in vitro* with animal cap cultures (explants of blastula ectoderm) treated with activin and retinoic acid (Uochi and Asashima, 1996). Over the last three years, our laboratory has identified over 30 genes with expression in the pronephric kidney of *Xenopus laevis* (Davies and Brändli, 1996; Brändli laboratory, unpublished data). This large collection of marker genes enables an extensive analysis of the molecular cascades acting during pronephric kidney development. Overall, the *Xenopus* embryo represents therefore a promising model for *in vivo* as well as *in vitro* studies to unravel the molecular and cellular basis of early kidney organogenesis.
4. **Aim of the thesis**

Pax genes have been implicated as key regulators of organ development in the vertebrate embryo. The aim of the present thesis is to gain further insight into the function of Pax-2/5/8 gene family members in early kidney organogenesis using the amphibian *Xenopus laevis* as a model. The objectives of the thesis are:

- to isolate and sequence cDNAs encoding all three *Xenopus* Pax-2/5/8 orthologues;

- to characterize the temporal and spatial expression of Pax-2/5/8 transcripts during *Xenopus* embryogenesis;

- to characterize the spectrum of alternative spliced Pax-2 transcripts in pronephric kidneys, animal cap cultures, and in whole embryos; and

- to assess the functional properties of different Pax-2 isoforms in pronephric kidney organogenesis and MHB development.

It is anticipated that the proposed studies will expand our understanding of how Pax genes control tissue formation and organogenesis. More specifically, a better knowledge of the molecular mechanisms underlying kidney organogenesis are of central importance in understanding the molecular basis of inherited renal diseases, such as the renal-coloboma syndrome. This will subsequently allow the development of novel diagnostic methods and serve as a starting point for improved therapeutic strategies.
5. Results

5.1. *Xenopus* Pax-2 displays multiple splice forms during embryogenesis and pronephric kidney development

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5.1.1. Abstract

Kidney organogenesis is initiated with the formation of the pronephric kidney and requires Pax-2 gene function. We report here the cloning and characterization of Pax-2 cDNAs from the frog *Xenopus laevis*, a model system suitable to study early kidney organogenesis. We show that expression of *Xenopus* Pax-2 (XPax-2) genes was confined to the nervous system, sensory organs, the visceral arches, and the developing excretory system. DNA sequencing of XPax-2 cDNAs isolated from head and pronephric kidney libraries revealed seven novel alternatively spliced Pax-2 isoforms. They all retain DNA-binding domains, but can differ significantly in their C-termini with some isoforms containing a novel Pax-2 exon. We investigated the spectrum of XPax-2 splice events in pronephric kidneys, animal cap cultures and in whole embryos. Splicing of XPax-2 transcripts was found to be extensive and temporally regulated during *Xenopus* embryogenesis. Since all investigated tissues expressed essentially the full spectrum of XPax-2 splice variants, we conclude that splicing of XPax-2 transcripts does not occur in a tissue-specific manner.
5.1.2. Introduction

The development of the vertebrate excretory system is characterized by the successive formation of three distinct kidneys, the pronephros, the mesonephros and the metanephros (Saxén, 1987). All three kidneys are derived from the intermediate mesoderm and appear in a precise temporal and spatial sequence during embryogenesis. The pronephros is established as the earliest and most anterior kidney and represents the simplest form of an excretory organ in vertebrates. Its structural organization consisting of two components, a tubular system and an excretory duct, has already been recognized in the early nineteenth century through anatomical studies of amphibian embryos (Müller, 1829). As with all kidneys, the basic excretory unit of the pronephric kidney is the nephron. Each nephron is initially composed of a glomerulus and a segmented tubular system. As development proceeds, the pronephric glomeruli fuse to a single vascularized glomus. Pronephric tubules are lined by diverse epithelial cell types and are connected at their proximal ends to the coelomic cavity via ciliated funnels (nephrostomes). Individual nephrons are linked together through connecting tubules, which fuse to a single broad collecting tubule that communicates with the pronephric (or Wolffian) duct (Fox, 1963; Fraser, 1950). The organization of pronephric nephrons is therefore very much like that of the more advanced mesonephric and metanephric kidneys. A major difference lies in the number of nephrons that comprises the different kidney types. While the metanephric kidney of humans can contain up to 1 million nephrons, the pronephric kidney of amphibians is typically composed of 2-3 nephrons. This simple structural organization makes the pronephric kidney an attractive system to study kidney organogenesis (Fox, 1963; Vize et al., 1995).

Maturation of the pronephric kidney into a functional excretory organ is restricted to larval stages of fish and amphibians. It mediates the excretion of metabolic end products from the blood, the reabsorption of ions, and the control of water balance, and is therefore essential for the survival of the embryo (Fox, 1963). In higher vertebrates, pronephric tubule differentiation remains rudimentary, but pronephric duct formation proceeds normally (Burns, 1955; Saxén, 1987). The pronephric duct and its derivatives participate in the induction of both the mesonephric and metanephric kidneys of vertebrate embryos (Burns, 1955; Herzlinger, 1995; Saxén, 1987). In amphibian embryos, the isolated pronephric duct has been shown to act in vitro as a strong inducer of mesonephric tubules if combined with explants of mesonephric mesenchyme (Etheridge, 1968). Similarly, induction of the metanephric kidney is dependent
on the ureteric bud, an epithelial diverticulum that forms from the caudal aspects of the pronephric duct. Ablation of the ureteric bud impairs metanephric kidney formation (Grobstein, 1955; Gruenwald, 1952; Saxén, 1987). While the role of the pronephric duct as an inducer of the mesonephric and metanephric kidneys is clearly established, it is currently not known whether a similar function is also necessary for proper pronephric kidney development. Since pronephric duct and tubules arise from separate but adjacent populations of primordial cells of the intermediate mesoderm (O'Connor, 1938; Vize et al., 1995), it is however conceivable that inductive interactions exerted by the pronephric duct could also promote pronephric tubule differentiation.

The molecular mechanisms underlying kidney organogenesis are in general still poorly understood. An ever growing number of molecules with supposed regulatory and signaling functions have been found to be expressed at various stages of kidney development (Davies and Brändli, 1996). The use of in vitro organ culture systems and genetic evidence provided by transgenic animal models has helped to define essential components of an emerging network of signaling pathways that regulate metanephric kidney development (Ekblom, 1996; Herzlinger, 1995; Lechner and Dressler, 1997). These components include growth factors of the Wingless/int (e.g. Wnt-4), transforming growth factor-β (e.g. GDNF), and bone morphogenetic protein (e.g. BMP-7) families; adhesion molecules (e.g. integrin α8), receptor tyrosine kinases (e.g. c-Ret), and transcription factors (e.g. BF-2, Emx-2 and WT-1). None of these gene products however appear to be absolutely necessary for normal pronephric and mesonephric kidney development. Thus, the nature of the molecules regulating early stages of kidney organogenesis remains still unresolved.

Transcription factors of the Pax class comprise a small, but important family of developmental control genes (Chalepakis et al., 1993; Mansouri et al., 1996; Stuart et al., 1994). Pax genes are defined by a 128-amino acid DNA binding domain, called the paired box domain, which is conserved in animal species ranging from nematodes to humans, and was originally identified in the Drosophila segmentation gene paired (Bopp et al., 1986). Based on sequence homology and the presence of class-specific amino acids in certain positions, the known paired domains can be divided into six different classes (Noll, 1993; Nornes et al., 1996; Walther et al., 1991). Each member of the family shows spatially and temporally restricted expression patterns during embryonic development, with the majority of Pax genes being expressed in the central nervous system and/or the paraxial mesoderm and its derivatives (Chalepakis
et al., 1993; Mansouri et al., 1996). Pax-2 is of particular interest with respect to kidney organogenesis. Pax-2 together with Pax-5 and Pax-8 comprise the class III Pax gene family (Walther et al., 1991). They contain an octapeptide motif and only a partial homeodomain. Pax-2 gene expression is associated with the earliest stages of kidney organogenesis (Dressler et al., 1990; Kadesh, 1992; Krauss et al., 1991; Mikkola et al., 1992; Püschel et al., 1992). Transcripts of Pax-2 are initially present in the pro- and mesonephric tubules, as well as in the pronephric duct and its derivatives. Later, Pax-2 expression is found in the condensing metanephric mesenchyme, its early epithelial derivatives, and in the collecting duct epithelium. Pax-2 transcripts are however never associated with the undifferentiated mesenchyme.

A number of mutations in the Pax-2 gene reported recently establishes a central role for Pax-2 in kidney organogenesis. In two human families, heterozygosity for Pax-2 mutations cause renal-coloboma syndrome (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). Patients display eye defects and kidney hypoplasia. The mutations appear to be loss-of-function mutations implying a dosage-sensitivity, haplo-insufficiency, for Pax-2. In mice, three strains with mutation in the Pax-2 gene have been reported. The Krd strain carries a large chromosomal deletion affecting several genes including the Pax-2 locus (Keller et al., 1994). Torres et al. (1995) have generated Pax-2-deficient mice by targeted mutagenesis. A spontaneous mouse mutant carrying the Pax-2$^{1}$Neu mutation was found to be identical to a previously described human Pax-2 mutation (Favor et al., 1996). All three mice mutants show that a partial loss of Pax-2 gene dosage results in similar renal defects as in humans (Favor et al., 1996; Keller et al., 1994; Torres et al., 1995). Animals homozygous for mutations in the Pax-2 gene are more severely affected and completely lack mesonephric and metanephric kidneys, ureters, and genital tracts (Favor et al., 1996; Torres et al., 1995). The pronephric duct develops only rudimentary, and then degenerates at the point when it should begin to interact with the nephrogenic mesenchyme. Outside the excretory system, defects are also seen with neural tube closure and the development of the optic nerve, the inner ear, and structures at the midbrain-hindbrain boundary (Favor et al., 1996; Sanyanusin et al., 1995a; Sanyanusin et al., 1995b; Torres et al., 1995). Recently, defects in nervous system, sensory organs and kidneys were reported for zebrafish strains harboring mutations in the no-isthmus (noi) gene which encodes a possible homologue of mammalian Pax-2 (Brand et al., 1996; Macdonald et al., 1997).
The nature of the kidney defects observed in homozygous mutant animals define Pax-2 as an essential component of the intermediate mesoderm necessary for normal pronephric kidney development. Being a transcription factor, Pax-2 may be directly controlling regulatory hierarchies of genes involved in this process. To address the mechanism of Pax-2 function in an experimental system suitable for studies on early kidney organogenesis, we have isolated cDNAs encoding seven novel isoforms of Pax-2 from *Xenopus* head and pronephric kidney libraries. We document here the spatial pattern of *Xenopus* Pax-2 gene expression during embryogenesis with an emphasis on pronephric kidney development. Further, we assess the complexity of XPax-2 isoform expression in embryos, animal cap cultures and developing pronephric kidneys. We found that alternative splicing of XPax-2 transcripts was temporally regulated. The profiles of XPax-2 isoform expression were however similar for all tissues examined. Our results indicate that the mechanism controlling alternative splicing of XPax-2 transcripts during early *Xenopus* embryogenesis is not regulated in a tissue-specific manner.
5.1.3. Results

Cloning and sequence analysis of cDNAs encoding Pax-2 homologues from Xenopus laevis

The reverse transcription-polymerase chain reaction (RT-PCR) was chosen as a strategy to isolate a Xenopus Pax-2 (XPax-2) specific nucleic acid probe. Invariant sequence motifs within the highly conserved paired domain of class III Pax genes were selected to derive degenerate oligonucleotide primers (Fig. 5A). Sequence analysis of 88 bacterial clones containing subcloned RT-PCR generated cDNA fragments allowed us to distinguish five different classes. The majority of the analyzed sequences (>95%) displayed highest homology to human Pax-2 and could be divided into two closely related subclasses. The two subclasses represent transcripts of two pseudoallelic genes for Pax-2 in Xenopus laevis, which we have termed XPax-2a and XPax-2b. The remaining sequences displayed highest homology to either Pax-5, Pax-6, or Pax-9. Next, we used XPax-2-specific cDNA fragments to screen a Xenopus stage 28-30 head cDNA library (Hemmati-Brivanlou et al., 1991) in order to obtain cDNA sequences harboring a complete open reading frame. Three full-length cDNAs were isolated: XPax-2a(1), XPax-2a(3), and XPax-2b(2). DNA sequencing allowed us to assign two cDNAs as transcripts of the XPax-2a gene, while the third was derived from XPax-2b. An alignment of the predicted protein products is shown in Fig. 5A. The three XPax-2 proteins are essentially collinear with each other over the first 300 amino acids. Within this region, their amino acid sequences are almost identical (5 amino acids differences between the XPax-2a and XPax-2b proteins; 98.3% identity). This degree of identity is characteristic for gene products of pseudoallelic Xenopus genes (Graf and Kobel, 1991). Vertebrate class III Pax genes are defined by the presence of three conserved sequence motifs: the paired domain, the octapeptide, and a partial homeodomain (Walther et al., 1991). All three XPax-2 proteins were found to display the same characteristic sequence motifs (Fig. 5A). A comparison of these motifs with the three human class III Pax proteins consistently indicated highest homology to human Pax-2 (Fig. 6). For XPax-2a(1), the amino acid identity was found to be 100% within the paired domain and the octapeptide motif (not shown), and 96% for the partial homeodomain. The identity in the C-terminal domain to human Pax-2 was with 87% somewhat lower, but still significantly higher than to human Pax-5 and Pax-8.
Fig. 5. Amino acid sequences of Xenopus Pax-2 cDNAs and comparison of exon composition with human Pax-2 and Pax-8 splice variants. (A) Alignment of the deduced amino acid sequences of full-length Xenopus Pax-2 isoforms. The amino acid sequences are shown in single letter code. Gaps introduced to maximize similarity in the sequences are shown as dots. Amino acids differing between pseudoallelic Xenopus Pax-2a and Pax-2b isoforms are boxed. The amino acid sequence motifs recognized by the degenerate PCR primers XP-1 and XP-2, respectively, are indicated by arrows. The paired domain is shown with a black line, the octapeptide motif with a double line and the partial homeodomain with a broken black line. Asterisks denote the approximate locations of introns that were identified by sequencing of the human Pax-2 gene (Sanyanusin et al., 1996). (Comparison of Xenopus Pax-2 splice variants with human Pax-2 and Pax-8 isoforms. Only sequences down-stream of the octapeptide motif are shown. hPax-2(E6) represents a splice variant of human Pax-2 containing exon 6 (Sanyanusin et al., 1996). hPax-2(E10) includes exon 10, which results in an alteration of the reading frame and thereby produces an alternative C-terminus (Ward et al., 1994). The sequence of human Pax-8a was taken from (Poleev et al., 1992). The exon organization of the coding sequence of human Pax-2 (Sanyanusin et al., 1996) is outlined beneath the sequence alignment. The exons of human Pax-2 are numbered from hE5 to hE12 and their boundaries are indicated by arrows. Note the gap between hE8 and hE9 generated by including XPax-2b(2) in the alignment. Human Pax-8a contains an alternatively spliced exon at the equivalent position.
Comparisons of Pax-2 proteins from zebrafish (previously referred to as Pax-B or \textit{pax}[zf-b]) and mouse to human Pax-2 revealed a similar degree of identity (Fig. 6). We conclude that XPax-2a and XPax-2b represent unambiguously \textit{Xenopus} homologues of the human Pax-2 gene.

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Fig. 6. Comparison of the deduced amino acid sequences of murine, \textit{Xenopus} and zebrafish Pax-2 with those of the human class III Pax proteins. The numbers indicate percent of identical amino acids within the domain examined. The paired domain covers a sequence motif of 128 amino acid residues (Bopp et al., 1986; Walther et al., 1991). The partial homeodomain is defined as 30-amino acid motif according to (Krauss et al., 1991). The C-terminal domain (137-140 amino acid residues in length) covers the entire region downstream of the partial homeodomain motif using human Pax-2 as a reference. To improve the alignment, intervening sequences generated by alternative splicing were removed from human Pax-8. The sequences were taken from the following sources: mouse Pax-2 (Dressler et al., 1990) with corrections according to (Adams et al., 1992); zebrafish Pax-2, originally described as \textit{pax}[zf-b] (Krauss et al., 1991); human Pax-2 (Eccles et al., 1992); human Pax-5 (Adams et al., 1992); human Pax-8 (Poleev et al., 1992).

Comparison of the C-terminal region of the XPax-2 cDNA sequences revealed that the extensive homologies in the open-reading frames are
interrupted by intervening nucleotide sequences downstream of the partial homeodomain (Fig. 5A). Relative to XPax-2a(1), XPax-2b(3) contains a 210 bp insertion, while XPax-2a(3) contains one of 99 bp. The extreme C-termini are again completely collinear. The intervening sequences do not alter the reading frame of the coding sequence. It is likely that these insertions represent alternative splice events during the maturation of XPax-2 transcripts. In humans, three Pax-2 variants generated by alternative splicing have been described (Eccles et al., 1992; Sanyanusin et al., 1996; Ward et al., 1994). Insertion of exon 6 between the octapeptide and the partial homeodomain generates a variant of human Pax-2, called here human Pax-2(E6) (Sanyanusin et al., 1996). Pax-2 isoforms homologous to human Pax-2(E6) have been described in zebrafish and mouse (Dressler et al., 1990; Krauss et al., 1991). Human Pax-2(E10) is generated by inclusion of exon 10. In this case, an alteration of the reading frame occurs and thereby produces a protein with an alternative C-terminus (Ward et al., 1994). We performed multiple sequence alignments to compare the variants of Xenopus Pax-2 with the known human Pax-2 isoforms (Fig. 5B). All three Xenopus Pax-2 isoforms lacked intervening sequences at a site equivalent to the exon 6 insertion in human Pax-2(E6). XPax-2a(1) was found to be homologous to the initially reported human Pax-2 protein (Eccles et al., 1992) which lacks exon 6 and exon 10 (data not shown). XPax-2a(3) is equivalent to human Pax-2(E10), with the exception that in Xenopus this alternative splicing event retains the original reading frame. XPax-2b(2) with its intervening sequence of 210 bp did not match any of the known vertebrate Pax-2 splice variants. The sequence alignment predicts that the alternative splice event occurs exactly at the junction of the human Pax-2 exon 8 and 9 (Fig. 5B). Transcripts of the related human Pax-8 gene are also subject to alternative splicing (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). Interestingly, a comparison of human Pax-8 and Xenopus Pax-2 isoforms revealed that XPax-2b(2) matched human Pax-8a including the novel intervening sequence (Fig. 5B). The alternatively spliced sequence of human Pax-8a is encoded by a separate exon (Kozmik et al., 1993; Poleev et al., 1995) and displays significant sequence homology to the Xenopus sequence (35.7% identity; 25 out of 70 amino acids). Taken together, the provided evidence suggests that the intervening sequence found in the C terminus of XPax-2b(2) might be encoded by a novel Pax-2 exon.
Results

Spatial expression of XPax-2 transcripts during early neural development, in sensory organs and visceral arches

We performed whole mount in situ hybridization using digoxigenin-labeled RNA probes (Harland, 1991) to determine the spatial expression of XPax-2 in the developing Xenopus embryo. Several different probes were constructed and tested in hybridizations with consistent results. These include probes covering the entire coding sequence, as well as such that exclude the conserved paired domain of each XPax-2 isoform (data not shown; see Experimental Procedures for details). For maximal sensitivity, only results from hybridizations with probes directed against the entire coding sequence of XPax-2a(1) are shown (Fig. 7). Expression of localized XPax-2 transcripts was first detected at the end of gastrulation and was thereafter seen throughout embryogenesis (Fig. 7). Transcripts were essentially confined to the nervous system (midbrain, hindbrain, spinal cord), sensory organs (optic vesicle and stalk, otic vesicle), the visceral arches, and the excretory system (pronephros, pronephric duct, rectal diverticulum, proctodaeum).

In the developing Xenopus nervous system, the earliest site of localized XPax-2 transcription was detected at stage 13 (early neurula) in the anterior 1/3 of the neural plate (Fig. 7A,B). XPax-2 expression was associated with two distinct wedge-shaped patches of cells flanking the midline, separated by a small region devoid of expression. In the course of neural tube closure, the two patches of XPax-2 expression converge towards the dorsal midline (Fig. 7C, D). These cells will ultimately form the posterior portion of the midbrain at the midbrain-hindbrain boundary (Fig. 7K). Expression of XPax-2 in the hindbrain became discernible at stage 24 and was initially confined to a region posterior to the otic vesicle (Fig. 7F). Later, expression started to spread also anteriorly. Finally, XPax-2-expressing cells were found throughout the hindbrain (Fig. 7I). Transversal sections at the level of the hindbrain-spinal cord junction revealed XPax-2-expressing cells at a ventrolateral location in the neural tube (Fig. 7M). A first indication of Pax-2 expression associated with neurons of the developing spinal cord became apparent in embryos of stage 19 (Fig. 7D). Expression was initially confined to the anterior 1/3 of the spinal cord (Fig. 7E). As spinal cord differentiation proceeds in the posterior direction, additional clusters of cells expressing XPax-2 were detected. By stage 32, clusters of XPax-2 expressing cells were found extending along all but the most posterior part of the spinal cord (Fig. 7H). Dorsal inspection of stained embryos revealed that the Pax-2 expressing cells appeared as two longitudinal columns (Fig. 7D). By sectioning transversally through the spinal cord, we were able to show that cells
expressing XPax-2 were confined to an intermediate position along the dorsal-ventral axis of the spinal cord (data not shown). Based on this location, these cells most likely represent interneurons (Hartenstein, 1993; Roberts and Clarke, 1982).
**Fig. 7.** Spatial expression of XPax-2 transcripts during early *Xenopus* embryogenesis. Whole mount *in situ* hybridizations were performed on albino embryos using digoxigenin-labeled antisense RNA probes. Hybridization events were visualized as alkaline phosphatase chromogenic reaction products. Embryos stained in whole mount were used for sectioning and were cut at 70 μm (L, M) and 100 μM (N). (A, B) Lateral and dorsal views of a stage 13 embryo. XPax-2 expression is seen in two wedge-shaped patches of cells in the anterior 1/3 of the neural plate. Expression is absent at the midline (arrowhead). (C) Lateral view of a stage 19 embryo. XPax-2 expression can be detected in the regions of the future midbrain-hindbrain boundary and the optic stalk (arrowhead). (D) Dorsal view of a stage 19 embryo. Two rectangular-shaped patches of XPax-2 expressing tissues which will contribute to the midbrain-hindbrain boundary flank the midline (arrowhead). Note that staining of XPax-2 can now also be detected in single neurons arranged in stripes on either side of the midline. (E) Lateral view of a stage 21 embryo. XPax-2 expression is associated with the region of the future optic stalk (os) and vesicle, the midbrain-hindbrain boundary (m-h), the otic vesicle (ov) and with cells in the spinal cord (sc). Note also the first appearance of staining in the pronephric anlage (pa). (F) Lateral view of a stage 24 embryo. XPax-2 expression can now also be detected in the furrows of the visceral arches (va), and in both compartments of the developing pronephric kidney, the pronephros (p) and the pronephric duct (pd). XPax-2 expression in the hindbrain (hb) is limited to the region posterior to the otic vesicle. (G) Lateral view of a stage 28 embryo. The proctodaeum (px) appears as a further tissue expressing XPax-2 transcripts. (H) Lateral view of a stage 32 embryo. Arrowheads indicate XPax-2 expression in the three nephrostomes of the pronephros. Note the appearance of the rectal diverticulum (rd) extending in anterior direction (open arrow) towards the posterior end of the pronephric duct (filled arrow). (I) Lateral view of a stage 36 embryo. The pronephric duct (filled arrow) and the rectal diverticulum (open arrow) are close to joining. Strong expression of XPax-2 is seen in the hindbrain and along the entire spinal cord. (J) Lateral view of a stage 39 embryo. The pronephric duct has fused with the rectal diverticulum (filled arrow). XPax-2 expression is detected in the entire excretory canal up to the point where fusion occurs with the cloaca (open arrow). The brown color in the eye is due to the onset of pigmentation. (K) Close-up view of a stage 39 embryo to illustrate details of XPax-2 expression in the optic stalk (filled arrow), the posterior end of the midbrain (open arrow), the otic vesicle (open arrowhead), and the hindbrain. Note also the extensive coiling of the pronephric tubules and the strong staining associated with the nephrostomes (filled arrowheads). (L) Transversal section through a stage 32 embryo which is cut anterior to the midbrain-hindbrain boundary. XPax-2 expression is confined to the ventral portion of the optic cup (arrowheads) and to furrows of the visceral arch (arrows). (M) Transversal section through a stained stage 32 embryo which is cut at the level of the hindbrain-spinal cord junction. Staining of the pronephroi (arrowheads) and of cells in the ventrolateral neural tube (arrows) is indicated. (N) Horizontal section through the visceral arches of a stage 28 embryo. The plain of section is below the eye. Arrowheads point to the expression in the furrows of the visceral arches. Embryos in shown in (A-K) were photographed uncleared, with the anterior ends of the embryo oriented to the left.
XPax-2 expression was also found to be associated with the development of two sensory organs, the eye and the ear. For the eye, XPax-2 transcripts were initially detected at stage 19 (late neurula) as a faint signal in the region of the future optic stalk and vesicle (Fig. 7C). By stage 21, expression was very prominent, and remained high during later stages as the optic stalk constricts and the optic vesicle invaginates. In transverse sections, XPax-2 transcripts were associated with the ventral region of the optic cup (Fig. 7L). In tadpole stage embryos, XPax-2 expression was gradually down-regulated, first in the optic cup, and then in the optic stalk (Fig. 7K). The onset of XPax-2 transcription in the developing ear correlated with otic placode induction (Fig. 7E). Invagination starts at stage 23, and separation from the epidermis is achieved by stage 28 (Nieuwkoop and Faber, 1956). XPax-2 expression remained associated with the otic vesicle throughout this period. By stage 39, XPax-2 expression was gradually down-regulated in the epithelium of the otic vesicle, but still remained in a patch of dorso-medially located cells (Fig. 7J, K).

Pax-2 expression in the context of visceral arch development has so far not been described. In *Xenopus*, we noticed three prominent stripes of XPax-2 expression which were associated with the visceral arches from stages 24 to 32 (Fig. 7F-H). Visceral arches, referred to as branchial or pharyngeal arches in higher vertebrates, are important for the development of the entire neck region in all vertebrate embryos (Carlson, 1996). The arches originate as bars of mesenchyme sculpted from the sides of the neck by pairs of visceral (or pharyngeal) pouches, which bulge out from the foregut endoderm. Where a visceral pouch approaches the overlying ectodermally-derived epidermis, it displaces the surrounding mesenchymal tissue and induces the formation of a furrow. In *Xenopus*, three visceral furrows have been described (Nieuwkoop and Faber, 1956). Each visceral arch is composed of epithelial layers of both ectodermal and endodermal origin, and contains premuscle mesenchyme of mesoderm origin, and a cranial nerve and a skeletal primordium of neural crest origin. We carried out horizontal sections to determine which of these tissues is associated with XPax-2 expression (Fig. 7N). XPax-2 transcripts were limited to the epidermal layer and highest concentrations were found in the three visceral furrows. Once embryos reached stage 36, the expression ceased (Fig. 7I).

**Expression of XPax-2 in the developing pronephric kidney**

The first morphological indication of the pronephric anlage is seen at stage 21 as a slight thickening of the somatic layer of the intermediate mesoderm below somites 3 and 4 (Nieuwkoop and Faber, 1956). This corresponds exactly
to the first time at which XPax-2 staining could be detected in the developing pronephric kidney. The embryos showed Pax-2 expression in a small extended stripe of tissue below the somites (Fig. 7E). By stage 24, the stripe of cells had extended in posterior direction with the future pronephros and pronephric duct becoming now discernible (Fig. 7F). The subsequent development of the pronephric kidney is characterized by the differentiation of the pronephros (into nephrostomes, tubular canaliculi and the collecting tube), the elongation of the pronephric duct, and the outgrowth of the rectal diverticulum. XPax-2 expression remained associated with the epithelial components of the pronephric kidney during each of these morphogenetic processes (Fig. 7G-K).

Transverse sections of stained embryos confirmed expression in the pronephros (Fig. 7M). Formation of the three nephrostomes begins at stage 28 and is completed by stage 34. This process was accompanied by a significant increase in XPax-2 expression as illustrated by three strongly stained spots in the pronephros (Fig. 7H,K). Expression of XPax-2 was also seen throughout the tubular system which was undergoing extensive coiling (Fig. 7K). The excretory canal is formed by fusion of two elongating epithelial components, the pronephric duct and the rectal diverticulum. Posterior elongation of the pronephric duct starts at stage 26, and continues until stage 36/37, when fusion with the rectal diverticulum occurs (Fig. 7H-J). The rectal diverticulum is formed from a bulge of the dorsal wall of the proctodaeum at stage 32 and grows in the anterior direction to make contact with the posterior growing pronephric duct. XPax-2 expression was detected starting from stage 28 in the region of the proctodaeum and became later also associated with the outgrowing rectal diverticulum (Fig. 7G, H). Expression of XPax-2 in the proctodaeum was subsequently down-regulated, first only ventrally and, later, also in dorsal aspects. By stage 39, XPax-2 expression remained in the epithelium of the excretory canal up to the point, where it fuses with the cloaca (compare Fig. 8H-J).

Isolation of cDNAs encoding novel XPax-2 isoforms from the developing pronephric kidney

The isolation of cDNAs encoding novel Pax-2 isoforms from a Xenopus head cDNA library prompted us to investigate alternative splicing of Pax-2 transcripts in the developing pronephric kidney. For this purpose, a cDNA library of XPax-2 cDNAs was prepared from RNA of stage 24 explants enriched for pronephroi (for dissection strategy, see Fig. 11A). For PCR amplification, primers XP-26 and XP-15 were chosen to anneal at the translational start site
and in the 3'-untranslated region, respectively. Both primers were designed to amplify cDNAs derived from XPax-2a and XPax-2b-encoding transcripts. The resulting cDNA libraries were analyzed systematically with the aim of identifying novel Pax-2 splice variants (see Materials and Methods for details).

Analysis of a total of 140 cDNA clones containing XPax-2 sequences led to the identification of six novel isoforms which were named XPax-2(4) to XPax-2(9). We determined the DNA sequences of XPax-2 cDNA clones representing each novel XPax-2 isoform class and compared them with the previously isolated XPax-2 isoforms (data not shown). Isoforms XPax-2(4), XPax-2(5) and XPax-2(7) were found to be derived from transcripts of the XPax-2a gene, while XPax-2(8) was from the XPax-2b gene. The cDNAs representing isoforms XPax-2(6) and XPax-2(9) were found to be hybrid clones consisting of sequences derived from both XPax-2 genes (see legend to Fig. 8). It is likely that these hybrid clones were generated during the PCR amplification process. Evidence for the existence of XPax-2 mRNA species homologous to isoforms XPax-2(6) and XPax-2(9) in Xenopus embryos and pronephric kidneys will be provided in later (Fig. 10).

In general, the generation of alternatively spliced transcripts can be understood based on the exon/intron organization of the corresponding gene. While the exon-intron structures of the two XPax-2 genes are currently not known, the genomic structure for human Pax-2 has been recently reported (Sanyanusin et al., 1996). Based on sequence comparisons of the Xenopus Pax-2 splice variants with human XPax-2 cDNAs of known exon compositions (Fig. 5; data not shown), it appears that the locations of introns have been conserved during evolution and it is likely that amphibia and humans share the same basic exon organization of their Pax-2 genes. Twelve exons have so far been identified in the human Pax-2 gene. The identification of a novel intervening sequence present in XPax-2b(2) and XPax-2a(4) suggests that Xenopus Pax-2 genes are composed of at least 13 exons. Based on this model, the structural organization of all XPax-2 isoforms isolated in this study has been diagrammed in Fig. 8. It appears that the diversity of XPax-2 isoforms is generated by alternative splicing of exons downstream of the DNA-binding paired domain. Exons 1, 2, 3, 4, 10, 12 and 13 were found to be common to all XPax-2 isoforms, while none appeared to contain sequences encoding exon 6. Finally, all described XPax-2 cDNAs retained the original reading frame despite being subject to extensive alternative splicing events.
Fig. 8. Schematic representation of Xenopus Pax-2 isoforms generated by alternative splicing. The structure of the Xenopus Pax-2 protein is shown schematically with the corresponding domain organization (PD, paired domain; OP, octapeptide; HD, homeodomain) indicated. The predicted exon structure is based on data from sequence comparisons of Xenopus Pax-2 isoforms (see Fig. 5) and on the exon organization of the human Pax-2 gene (Sanyanusin et al., 1996). The positions of the introns are indicated by arrows. Exons are numbered from 1 to 13. The position of sequences in exons 1, 3, 4, 7, 8, 10 and 13, respectively, used to derive primers for RT-PCR are identified by arrows which are labeled with the name of the primer. Below, cDNAs encoding different splice variants of XPax-2 are drawn diagrammatically. Splice variants XPax-2(1), XPax-2(2), and XPax-2(3) represent full-length cDNAs isolated by screening from a Xenopus head cDNA library. The cDNAs encoding XPax-2(4) to XPax-2(9) were isolated from a cDNA library generated from pronephric kidney RNA. The sequenced cDNAs clones were derived from transcripts of the XPax-2a gene in the case of XPax-2(1), XPax-2(3), XPax-2(4), XPax-2(5) and XPax-2(7) and for XPax-2(2) and XPax-2(8) from the XPax-2b gene. For hybrid clone XPax-2(6), exons 1, 2, 3 and 4 were from XPax-2b and exons 7, 8, 10, 11, 12 and 13 of XPax-2a. For hybrid clone XPax-2(9), exons 1, 2, 3, 4 and 10 were derived from the XPax-2a gene, while exons 12 and 13 originated from the XPax-2b gene.
Alternate use of consensus 3' splice acceptors generates additional diversity among XPax-2 mRNAs

Canonical 3' splice acceptor sequences are characterized by an invariant AG dinucleotide at the -2 and -1 positions of the intron, and are usually preceded by a C or U at the -3 position (Stephens and Schneider, 1992). It has been previously shown that distinct splice forms of Pax mRNAs can be attributed to the use of alternate 3' splice acceptors (Kozmik et al., 1993; Poleev et al., 1995; Vogan et al., 1996). DNA sequence comparisons revealed evidence that similar alternative splicing events occur in XPax-2 transcripts. The N-terminus of isoform XPax-2b(8) differed from XPax-2b(2) by the absence of three nucleotides, CAG, at the junction of exon 1 and 2 (Fig. 9A). The observed deletion is most likely due to alternative splicing to an AG acceptor splice site present in exon 2 as illustrated in Fig. 9. The deduced amino acid sequence of XPax-2b(8) predicts that the proline and glycine residues are replaced by a single arginine residue. This splicing event was not unique to XPax-2b(8), since the exact same CAG trinucleotide was found to be deleted in the cDNA encoding the XPax-2(9) isoform (data not shown). Comparison of the C termini of XPax-2a(7) and XPax-2a(1) revealed that XPax-2a(7) contains an insertion of nine nucleotides located between exon 11 and 12 (Fig. 9B). The insertion probably represents sequences derived from intron 11. A consensus 3' splice site at the boundary with exon 12 could be identified. We suggest that the use of a second 3' splice acceptor flanking the 5' end of the insertion is responsible for the generation of the insertion. The confirmation of this hypothesis will however have to await sequencing of the exon-intron junctions of the XPax-2 genes.

Finally, sequencing of a number of additional XPax-2 cDNA clones revealed the presence of a further consensus 3' splice sequence present in exon 13 (Fig. 9C). Use of this site results in the splicing out of a 19-nucleotide sequence of exon 13. This leads to a shift in the reading frame and gives rise to a Pax-2 isoform with distinct C terminus (Fig. 9D). In summary, our results suggest that differential utilization of consensus 3' splice acceptor sites present in exons 2 and 13, and presumably in intron 11, generates additional diversity of XPax-2 transcripts.
Fig. 9. Novel isoforms of XPax-2 generated by alternate use of 3' splice acceptors. Invariant AG dinucleotides which can serve as consensus 3' splice sites are underlined. Nucleotide sequences altered through use of such alternative 3' splice acceptors are boxed. The new open reading frames are shown below. Amino acids that differ from the original deduced amino acid sequence are indicated with bold letters. The positions of the introns are indicated by arrows and are based on the structure of the human Pax-2 gene (Sanyanusin et al., 1996). (A) XPax-2b(8) contains a three-nucleotide deletion within exon 2. The nucleotide and amino acid sequences of XPax-2b(2) are shown below. The nucleotide sequence deleted in XPax-2b(8) is boxed. (B) XPax-2a(7) contains an insertion of nine nucleotides located between exon 12 and 13. The sequence is presumably derived from intron 11 and has a consensus 3' splice site. For comparison, the equivalent region in XPax-2a(1) is given below. (C) Deletion of nineteen-nucleotide sequence within exon 13 creates a splice variant of XPax-2 with an altered C-terminus. Below, the nucleotide sequence of XPax-2a(1) is shown to outline the nineteen-nucleotide deletion identified in several XPax-2 cDNA clones. (D) Comparison of the deduced amino acid sequences at the extreme C-termini of XPax-2a(1) and XPax-2 splice variants (below) containing the nineteen-nucleotide deletion within exon 13. Asterisks represent stop codons.
Results

Temporal regulation of alternative splicing of XPax-2 transcripts in the early Xenopus embryo

We used RT-PCR to address whether expression of XPax-2 isoforms is regulated during Xenopus embryogenesis. For PCR, several sets of primer pairs were selected based on the proposed exon organization of the XPax-2 genes (Fig. 8). All PCR primers were designed to amplify cDNAs derived from transcripts of both XPax-2 genes. The RT-PCR assay was carried out in conjunction with Southern blotting and hybridization to visualize amplification products encoding XPax-2 sequences. The identity of specific bands was determined in comigration experiments with amplification products of known exon composition (Fig. 10C, D; and data not shown). In some cases, amplification products were also verified by cloning and DNA sequencing (e.g. products marked by asterisks and filled circles in Fig. 10D). For a given XPax-2 isoform, the amplification products of the two pseudoallelic forms are of equal size and can therefore not be distinguished in the assay used here. We will therefore not maintain a distinction between the two pseudoallelic XPax-2 genes in describing the exon composition of the observed amplification products. We also want to stress that RT-PCR was performed "semi-quantitatively" allowing us to assess the presence or absence of specific XPax-2 transcripts rather than determining precise expression levels.

Alternative splicing in the paired domain can dramatically alter the DNA binding affinity of Pax proteins as described for Pax-3, Pax-5 and Pax-6 (Epstein et al., 1994b; Vogan et al., 1996; Zwollo et al., 1997). To determine whether similar alternative splicing events can occur in the paired domain of XPax-2, we selected for RT-PCR primers XP-25 and XP-31 directed against sequences in exon 1 and exon 3, respectively. All cloned XPax-2 cDNAs contain exons 1, 2 and 3 (see Fig. 8), and generate in PCR experiments amplification products of 380 bp (data not shown). RT-PCR experiments using RNA isolated from embryos ranging from unfertilized eggs to stage 34 tadpole embryos consistently resulted in a single amplification product composed of exons 1, 2 and 3 (Fig. 10A). Evidence for maternal transcripts present in unfertilized eggs and in blastula stage embryos was obtained upon overexposure of the blot (data not shown). The vast majority of XPax-2 transcripts were however detected in embryos from stage 12 on with expression levels remaining essential constant throughout the embryonic stages tested (Fig. 10A). These observations are consistent with our in situ hybridization studies, which place the onset of spatially localized XPax-2 transcription to mid-gastrula stages (Fig. 7).
Results

Fig. 10. Temporal regulation of alternative splicing of XPax-2 transcripts during early Xenopus embryogenesis. Schematic representations of the XPax-2 protein are shown in panels A-D. Conserved sequence motifs (PD, paired domain; OP, octapeptide; HD, partial homeodomain) are outlined, and arrows mark the relative positions of the sequences recognized by the primers used for RT-PCR (see also Fig. 8). Total RNA was extracted from unfertilized eggs (E) and embryos of the indicated stages, and assayed for the expression of XPax-2 splice variants by RT-PCR. Amplification products were separated on agarose gels, transferred onto nylon membranes and hybridized with appropriate radiolabeled XPax-2 probes. The migration positions of size standards are shown on the left of the autoradiographs. On the right, selected amplification products are identified by arrowheads. Their exon composition was determined by comparison with XPax-2 cDNAs of known exon composition (autoradiographs on the right of C and D) and by sequencing of the cloned amplification products. The numbering of exons is based on the model in Fig. 8. (A) Alternative splicing in the paired domain region. Primers XP-25 and XP-31 directed against sequences in exon 1 and 3, respectively, were used for the RT-PCR analysis. A single amplification product with a structural organization consisting of exons 1, 2 and 3 was detected. (B) Alternative splicing in the region of the octapeptide motif. Primers XP-59 and XP-11 are directed against sequences in exon 4 and 7, respectively. The autoradiograph displays the temporal expression profile of alternatively spliced XPax-2 transcripts in the early embryo. (C) Alternative splicing between exons 4 and 10. Primers XP-59 and XP-63 are used for PCR amplification. The autoradiograph on the left displays the spectrum of alternative splice variants in the developing embryo. The autoradiograph on the right compares the amplification products generated with XPax-2 cDNA templates of defined exon composition to those detected in the developing embryo. (D) Alternative splicing in the C-terminal region. Primers XP-9 and XP-24 are directed against sequences in exon 8 and 13, respectively. The autoradiograph on the left displays the spectrum of alternative splice variants in the developing embryo. Amplification products generated by alternate use of 3' splice acceptors present in intron 11 or exon 13 are marked by asterisks or filled circles, respectively. The autoradiograph on the right compares the amplification products generated with XPax-2 cDNA templates of defined exon composition to those detected in the developing embryo. (E) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with ornithine decarboxylase (ODC) specific primer pair ODC-1/ODC-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers.

The next set of PCR primers was chosen to investigate alternative splice events in the region of the octapeptide motif. In particular, we were interested in investigating whether we could detect XPax-2 transcripts containing the predicted exon 6, for which evidence exists in zebrafish, mouse and human (Dressler et al., 1990; Krauss et al., 1991; Sanyanusin et al., 1996). Primers XP-59 and XP-11 were designed to recognize sequences within exon 4 and exon 7, respectively (Fig. 8). RT-PCR experiments allowed us to identify two amplification products (Fig. 10B). The larger amplification product was shown to
Results

consist of exons 4, 5, and 7, while the smaller one contained exons 4 and 7 (data not shown). Both amplification products could not be detect in pre-gastrula embryos, but were present in embryos from stage 12 on. Together with evidence presented below (Fig. 10D), it appears that XPax-2 transcripts composed of exon 6 are not expressed during early Xenopus embryogenesis.

The exons 4 and 10 are common to all XPax-2 cDNAs described in this study (Fig. 8). We therefore chose primers XP-59 and XP-63 to investigate splicing between these exons. The RT-PCR reactions revealed at least eight distinct XPax-2 bands and for five of the eight amplification products the exon composition was determined (Fig. 10C; and data not shown). None of the amplification products appears to contain exon 6. The majority of the amplification products were detected in gastrula stage and older embryos. Two amplification products, however, were detected also in pre-gastrula embryos. They were found to be composed of exons 4 and 10, and exons 4, 5, and 10, respectively. Evidence for maternal expression of XPax-2 transcripts had also been found with primers annealing to exons 1 and 3, respectively (see text to Fig. 10A). We conclude that maternal transcripts of XPax-2 contain either exons 1, 2, 3, 4 and 10 or exons 1, 2, 3, 4, 5 and 10. Only isoforms XPax-2(7), XPax-2(8) and XPax-2(9) have exon compositions consistent with maternal mRNA expression. Further, XPax-2 isoforms (1) to (6) are expressed exclusively as zygotic mRNAs in the early Xenopus embryo.

The different XPax-2 isoforms diverge mainly in their C-terminal domains indicating that this region is possibly most susceptible to alternative splice events. We therefore selected primers XP-9 and XP-24 to investigate how alternative splicing affects the C-terminal domain between exon 8 and 13 (Fig. 8). This primer pair will detect only expression of strictly zygotic XPax-2 mRNAs. RT-PCR experiments revealed a complex pattern of amplification products ranging in size from 270 to 580 bp (Fig. 10D). Amplification products with exon compositions corresponding to cloned XPax-2 isoforms were readily identified (Fig. 10D, right panel). Two minor bands (identified by asterisks and filled circles in Fig. 10D) were found to be flanking the amplification products containing exons 8, 10, 11, 12 and 13 and exons 8, 10, 12 and 13. We demonstrated by cloning and DNA sequencing that they represent transcripts generated by the alternate use of consensus 3' splice sites present in intron 11 and exon 13, respectively, as described earlier (see Fig. 9B, C). None of the amplification products were detected before mid-gastrula stages indicating that isoforms XPax-2(1) to XPax-2(6) are not expressed as maternal transcripts. Most of the detected amplification products are not subject to significant
regulation of expression levels between gastrula stages and stage 34. The largest amplification product comprising of all exons from 8 to 13 represents an exception, since it is predominantly detected in gastrula and neurula stage embryos. In summary, we have been able to define two classes of XPax-2 mRNAs. A first group consisting of strictly zygotic XPax-2 mRNAs and second one comprised of those transcripts which display, in addition, maternal expression.

Expression of XPax-2 splice variants in the developing pronephric kidney

Isoforms XPax-2(1) to (3) were identified in a cDNA library prepared from heads of stage 28-30 embryos, while XPax-2(4) to (9) were isolated from cDNA libraries of stage 24 pronephric explants. The question therefore arises whether expression of specific XPax-2 isoforms is restricted to the developing pronephric kidney. We employed the RT-PCR approach using now RNA prepared from embryos dissected into heads and explants enriched for pronephroi. Two embryonic stages were chosen for the dissections with the purpose of representing an early (st. 24) as well as an advanced stage (st. 36) of pronephric kidney development. Particular care was taken to assure that pronephric explants were not contaminated with neural tissues (e.g. spinal cord). Head explants contained most of the XPax-2 expressing tissues with the exception of spinal cord and pronephric kidneys (Fig. 11A).

We carried out RT-PCR experiments using primers XP-59 and XP-63 to monitor splicing events between exons 4 and 10, which are found in all described XPax-2 isoforms. Using cDNAs from pronephric kidney explants as templates, we were able to detect at least eight distinct amplification products (Fig. 11B). The exon composition of five bands could be assigned. They represent the most abundant amplification products. Three amplification products remain currently uncharacterized. In most cases, the expression levels of the amplification products did not change between stage 24 and stage 36 pronephroi. Interestingly, the profiles of amplification products observed with cDNAs from pronephric explants were essentially identical to those obtained with cDNAs from heads with the exception of an amplification product composed of exon 4 and 10. This band was found predominantly in older pronephroi, but longer exposures of the blot revealed faint signals in stage 24 pronephroi and in head explants of both embryonic stages (data not shown).
Results

Fig. 11. Alternative splicing of XPax-2 transcripts in the developing pronephric kidney. (A) Outline of the dissection strategy. To illustrate the distribution of XPax-2 expressing tissues, embryos of stage 24 and 36, respectively, stained by in situ hybridization are shown. The dissection planes are indicated with black lines. Heads (h) and explants enriched in pronephric tissue (p) were used for RNA isolation, and subsequent RT-PCR analysis. Head explants contain brain tissues, sensory organs, and visceral arch material. (B) Identification of alternative splice variants using primer pair XP-59/XP-63. Head (h) and pronephric (p) explants were analyzed by RT-PCR. The autoradiograph is shown with the size markers on the left. Amplification products verified by DNA sequencing are identified by arrowheads and their exon compositions are shown. (C) Identification of alternative splice variants using primer pair XP-9/XP-24. (D) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with histone H4 specific primer pair H4-1/H4-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers. The limitations of histone H4 as a control for pronephric RNA preparations are discussed in the result section.
Results

To control for equal amounts of RNA in cDNA synthesis reactions, the expression of commonly used control genes such as ornithine decarboxylase (ODC), elongation factor 1α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or histone H4 was monitored. We found that all of these genes were suitable standards, if the RNA preparation originated from embryonic heads. However, if RNA was obtained from pronephric kidney explants, expression of control genes was either not detectable (ODC, EF-1α, and GAPDH; data not shown) or in the case of histone H4 was found to be at lower levels than expected (Fig. 11D). Similar observations have been reported previously, e.g. for cells of the thyroid (Savonet et al., 1997). We therefore believe that in the absence of a better standard the spectrophotometric measurement of total RNA for pronephric tissues represents the only reliable method of quantification. Taken together, our experiments have so far indicated that splicing of XPax-2 transcripts generates in the pronephric kidney a similar spectrum of isoforms as seen in head explants. Indeed, the expression profiles were found to be indistinguishable to those obtained with cDNAs from whole embryos of the corresponding stages (compare with Fig. 10D).

In a separate set of RT-PCR experiments, we also used primers to sequences in exons 8 and 13 located in the C-terminal domain of XPax-2. As shown in Fig. 10C, amplification products for all XPax-2 isoforms were present in both tissues analyzed. In younger pronephroi, the expression levels of amplification products containing exon 9 were found to be rather low. Higher levels of amplification products containing exon 9 were however detected in older pronephroi, and the presence of XPax-2 transcripts in pronephric kidney was also confirmed by in situ hybridization using a probe consisting solely of exon 9 (data not shown). Finally, we also carried out RT-PCR experiments using primers XP-59 (exon 4) and XP-24 (exon 13). Again, we could not discern any clear difference between XPax-2 isoforms expressed in the head or the pronephric kidney (data not shown). In summary, the evidence present here argue strongly against the expression of a unique complement of XPax-2 splice variants in the developing pronephric kidney. We can however not be excluded that the relative abundance of splice variants might vary during pronephric kidney development.
Results

Induction of XPax-2 transcription in animal cap explants by growth factors

In Xenopus, animal cap cultures represent a convenient way to induce different tissues types in vitro (Asashima, 1994; Dawid, 1994; Kadesh, 1992; Kessler and Melton, 1994; Slack, 1994). For example, the growth factor activin can induce animal cap explants to differentiate in a dose-dependent manner into neural tissue and almost all mesodermal tissues. On the other hand, treatment with basic fibroblast growth factor (bFGF) promotes mainly mesoderm of ventrolateral type. Retinoic acid by itself does not induce mesoderm in animal cap explants, but it can, if added in combination with activin, promote the induction of pronephric tubules at a very high frequency (Moriya et al., 1993; Uochi and Asashima, 1996). We therefore decided to investigate the regulation of XPax-2 expression and alternative splicing under in vitro conditions in animal cap cultures.

Animal caps were excised from stage 8-9 embryos, and treated with either activin, bFGF, retinoic acid, or a combination of activin and retinoic acid. Once control embryos reached stage 28, the caps were analyzed for the expression of XPax-2 by RT-PCR as well as by whole mount in situ hybridization (Fig. 12). Untreated animal caps or caps incubated with retinoic acid alone were unable to activate XPax-2 gene transcription as illustrated in RT-PCR experiments using primers pairs XP-59/XP-63 and XP-9/XP-24, respectively (Fig. 12A, B). Strong induction of XPax-2 transcription was seen in the presence of activin, while bFGF was found to be a much less potent inducer. The profile of amplification products was however very similar for both growth factor and mirrored the situation described earlier for whole embryos (compare Fig. 12A, B with Fig. 10C, D). Interestingly, the inclusion of retinoic acid in the activin-treated cultures neither changed the pattern nor the extent of XPax-2 splicing (Fig. 12A, B). The induction of Pax-2 transcripts in animal cap cultures could also be confirmed by in situ hybridization (Fig. 12D). Animal caps responded to activin by extensive elongation and displayed patches of strong XPax-2 staining. Retinoic acid, in combination with activin, significantly suppressed the elongation process, but still allowed expression of XPax-2 transcripts. Despite the fact that all treatments used here have been described to induce different spectra of tissues in animal caps (Green et al., 1990; Moriya et al., 1993; Uochi and Asashima, 1996), we were unable to detect significant differences in the splicing of XPax-2 transcripts. Based on our in vivo and in vitro experiments, we conclude that splicing of XPax-2 transcripts is not regulated in a tissue-specific manner during early Xenopus embryogenesis.
Fig. 12. Induction of XPax-2 splice variants in animal cap explants. Embryos were cultured until stage 8-9, at which the upper part of the animal hemisphere (animal cap) was removed. The animal caps were treated for 12 hrs at 22°C with either activin (10 ng/ml), retinoic acid (RA, 10 μM), basic FGF (bFGF; 100 ng/ml) or a combination of activin and RA. Control explants were cultured in the absence of any factors. Expression of XPax-2 splice variants was assayed by RT-PCR (panel A-C) and by whole mount in situ hybridization (panel D) once reference embryos reached stage 28. In panels A and B, schematic representations of the XPax-2 protein are shown to illustrate the RT-PCR strategy. Autoradiographs is shown with the size markers on the left. (A) Identification of alternative splice variants using primer pair XP-59/XP-63. (B) Identification of alternative splice variants using primer pair XP-9/XP-24. (C) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with elongation factor-1α (EF-1α) specific primer pair EF1α-1/EF1α-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers. (D) Induction of XPax-2 expressing tissues in animal cap explants. Animal caps were incubated either under control conditions or in presence of added factors (activin alone; activin and RA) as described above. Note the absence of XPax-2 transcripts in control animal caps.
5.1.4. Discussion

The simple organization together with its role as an inducer of the mesonephric and metanephric kidney makes the pronephric kidney attractive for studies on the molecular and cellular mechanisms that control kidney organogenesis. In the present study, we report the isolation and characterization of cDNAs for Pax-2, a possible regulator of early kidney organogenesis, from the frog *Xenopus laevis*. We found that XPax-2 gene expression is associated with all epithelial structures of the developing pronephric kidney from the onset of morphogenesis. During this developmental process, XPax-2 transcripts are extensively spliced as illustrated by the isolation of cDNAs encoding multiple novel Pax-2 isoforms from pronephric kidney libraries. An analysis of XPax-2 transcripts from pronephroi and other embryonic tissues revealed that alternative splicing was not regulated in a tissue-specific manner. The implications of our findings will be discussed below.

Evolution and genomic organization of vertebrate class III Pax genes

Three genes, Pax-2, Pax-5 and Pax-8, comprise currently the class III Pax gene family in vertebrates (Noll, 1993; Nornes et al., 1996; Walther et al., 1991). Class III Pax genes arose by two independent gene duplications from a common ancestral gene (Noll, 1993). Based on sequence homologies and phylogenetic tree analysis, it is believed that a first duplication event produced Pax-8 and an ancestor gene with properties of both Pax-2 and Pax-5. A second, more recent duplication event generated Pax-2 and Pax-5. Both duplication events apparently occurred in the course of vertebrate evolution after the separation of the lines leading to fish and higher vertebrates (Noll, 1993). Supporting this hypothesis, zebrafish Pax-2 (previously referred to as Pax-B or *pax[zf-b]*) represents the only class III Pax gene identified in fish so far. The complexity of the class III Pax gene family in amphibians was not known up to now. The Pax cDNAs isolated in this study were shown by sequence comparison and expression pattern to be clearly *Xenopus* homologues of mammalian Pax-2 genes. In addition, we have also isolated cDNAs encoding a *Xenopus* Pax-5 homologue (N. Heller and A.W. Brändli; manuscript in preparation). We currently lack evidence for the existence of *Xenopus* Pax-8 homologue. Since the duplication event that generated Pax-2 and Pax-5 is considered to be the evolutionary younger event, the possibility exists that the *Xenopus* genome might also harbor Pax-8 genes. We therefore
believe that the currently known complexity of class III Pax genes was established before the separation of amphibians and higher vertebrates in evolution.

The common evolutionary origin of the class III Pax genes is also reflected in their genomic organization. All the known intron positions are strictly conserved between human Pax-2, Pax-5 and Pax-8 (Busslinger et al., 1996; Kozmik et al., 1993; Poleev et al., 1995; Sanyanusin et al., 1996). Although the genomic organization of the *Xenopus* Pax-2 genes are currently not known, the evidence from our studies on alternative splicing of XPax-2 transcripts are consistent with this notion. For example, sequence comparisons revealed that XPax-2a(1) and XPax-2a(3) represent *Xenopus* homologues of previously reported human Pax-2 splice variants (Fig. 5B). Furthermore, all XPax-2 isoforms reported here can be explained using the exon organization of the human Pax-2 gene as a paradigm (Fig. 8).

Two cDNAs, XPax-2b(2) and XPax-2a(4), are generated by inclusion of a novel intervening sequence (Fig. 5B, 8). Interestingly, the human Pax-8 gene contains a homologous sequence encoded by an alternatively spliced exon (identified as exon 8 (Poleev et al., 1995) or exon 9 (Kozmik et al., 1993), respectively). It is therefore likely that the novel XPax-2 intervening sequence is also encoded by an exon, which would represent a novel vertebrate Pax-2 exon. Based on this observation, we propose that the coding sequences of the *Xenopus* Pax-2 can be composed of up to 13 exons (Fig. 8). We have so far been able to obtain nucleotide sequence information on all exons, but exon 6. Isolation of genomic clones of XPax-2 should resolve this remaining open question.

**Pax genes in visceral arch development**

Analysis of XPax-2 expression during *Xenopus* embryogenesis revealed that transcripts were associated with the developing nervous system, sensory organs and the excretory system (Fig. 7). This expression pattern is essentially identical to that reported for Pax-2 homologues in zebrafish (Krauss et al., 1991; Mikkola et al., 1992; Püschel et al., 1992) and mice (Dressler et al., 1990; Nornes et al., 1990; Püschel et al., 1992) and indicates that the mechanisms controlling tissue-specific regulation of Pax-2 gene expression have been conserved during vertebrate evolution. In addition, we identified Pax-2 expression in the ectodermally derived furrows of the visceral arches (Fig. 7N). This novel site of Pax-2 expression has possibly been overseen in previous studies of zebrafish and mouse embryos.
What could be the role of Pax-2 in visceral arch development of the vertebrate embryo? In water-living embryos of lower vertebrates, the furrows and pouches of the visceral arches fuse to form the visceral clefts or gill slits. It is conceivable that Pax-2 could have a role in this process by regulating the fusion process. Other morphogenetic processes leading to fusion processes, such as optic fissure and neural tube closure, have been shown in the past to depend on Pax-2 gene function (Favor et al., 1996; Torres et al., 1996). In the mammalian embryo, only the first furrow gives rise to a recognizable structure (Carlson, 1996). It persists as the outer ear, while the other furrows are overgrown. The tympanic membrane (or ear drum) is formed where the first furrow meets the first pharyngeal pouch. By extension, Pax-2 could be necessary for some aspects of ear development in the mammalian embryo. Recent studies of mouse mutants have shown that Pax-2 is required for the development of the auditory system in the inner ear (Torres et al., 1996). Whether a lack of Pax-2 expression also affects the morphogenesis of the outer ear or the formation of the tympanic membrane remains to be established.

Expression of XPax-2 in the developing pronephric kidney

We have demonstrated that XPax-2 expression is associated with developing pronephric kidney (Fig. 7). How does its expression correlate with the major events of organogenesis: induction, determination, morphogenesis, and differentiation? The time point at which pronephric kidney induction occurs in the amphibian embryo is unknown, but the prospective pronephric area has been mapped in gastrulating embryos to a region of the marginal zone ventrolateral to the blastopore (Pasteels, 1942). Determination occurs by the midneurula stage, when the prospective pronephric area will form pronephric tubules, even when transplanted to heterotopic sites (Fales, 1935). As morphologically distinguishable structure, the pronephric anlage becomes first apparent with the completion of neurulation. In Xenopus embryos, it appears as a homogenous bulge in the mesoderm directly ventral to the somites at approximately embryonic stage 21, which correlates precisely with the first detectable expression of XPax-2 (Fig. 7E). The timing of XPax-2 expression makes it therefore rather unlikely that XPax-2 has a central role in the determination process, but rather suggests a role in pronephric kidney morphogenesis and/or differentiation. This view is supported by studies in zebrafish and mice deficient for Pax-2, where the earliest defects in the excretory system are associated with the elongation of the pronephric duct (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995).
A poorly understood aspect of pronephric kidney development is the contribution of the rectal diverticulum to the formation of the excretory canal. In the tailbud stage *Xenopus* embryo, a pair of rectal diverticuli form by evagination from the dorsal wall of the proctodaeum. They elongate in the anterior direction to fuse with the posterior growing pronephric ducts. XPax-2 was found to be expressed in both the proctodaeum and the extending rectal diverticuli, demonstrating unequivocally their contribution to the establishment of the excretory canal (Fig. 7G-J). To our knowledge, XPax-2 represents the first molecular marker found to be expressed in the rectal diverticuli.

**Alternative splicing of class III Pax gene transcripts**

The cloning of Pax-2 cDNAs from zebrafish (Krauss et al., 1991), mouse (Dressier et al., 1990) and human (Eccles et al., 1992; Sanyanusin et al., 1996; Ward et al., 1994) had led to the identification of three Pax-2 splice isoforms. In *Xenopus*, we have isolated through screening of head and pronephric kidney cDNA libraries clones for nine distinct isoforms (Fig. 8). Two of these isoforms, XPax-2a(1) and XPax-2a(3), were homologous to previously identified mammalian isoforms. Thus, alternative splicing of Pax-2 transcripts apparently generates at least ten distinct vertebrate Pax-2 isoforms. We believe that the number of expressed splice variants generated *in vivo* is in fact even higher. For one, the exon compositions of a number of amplification products are still unknown (see Fig. 10B, C). For the other, we have provided evidence that the diversity of Pax-2 transcripts is further increased by through alternate use of consensus 3' splice acceptors (Fig. 9). These alternative splicing events can either lead to small deletions or insertions of one to three amino acids residues, while still preserving the original reading frame, as observed with XPax-2b(8) and XPax-2a(7). In the case where exon 13 is affected, splicing can even generate Pax-2 isoform with an alternative C-terminus (Fig. 9C, D). A Pax-2 isoform with an alternative C-terminal domain has been previously also reported for human Pax-2 (Ward et al., 1994). Transcripts of the related Pax-5 and Pax-8 genes are also modified by alternative splicing. For Pax-5, transcription from two distinct promoters results in splicing of two alternative 5' exons, exon 1a and exon 1b, to the common coding sequence (Busslinger et al., 1996). In addition, three novel splice forms have been recently found to be expressed during B-cell development (Zwollo et al., 1997). For the Pax-8, a total of seven splice variants have been identified (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). Alternative splicing of class III Pax gene transcripts is therefore not uncommon, but our studies have revealed that
XPax-2 transcripts are subjected to a degree of alternative splicing that had previously not been anticipated.

**Functional implications of Pax-2 splicing**

Transcripts that either contain or lack particular exons may have entirely different functions at the protein level (Foulkes and Sassone-Corsi, 1992; McKeown, 1992). Pax proteins are thought to function as transcription factors by being able to bind to specific DNA elements of target genes and to act as transcriptional activators. Alternative splicing has been found to affect both aspects of Pax protein function. Introduction of an additional exon into the paired domain of Pax-6 modifies the structure and DNA-binding properties of the protein (Epstein et al., 1994b), while deletion of an exon in the Pax-5 paired domain abolishes DNA binding completely (Zwollo et al., 1997). An alternative mechanism has been described for Pax-3 and Pax-7, where alternate use of consensus 3' splice sites results in the removal of a single glutamine residue generating isoforms with increased affinity to target DNA sequences (Vogan et al., 1996). Structural motifs mediating transactivation are located in the C-terminal domain of Pax proteins, a region that is preferentially modified by alternative splicing. For Pax-8, several isoforms carrying alternative C termini have been described to harbor distinct transactivation abilities (Dehbi and Pelletier, 1996; Kozmik et al., 1993; Poleev et al., 1995). Similar results were recently reported for isoforms of Pax-9 (Nornes et al., 1996).

How does alternative splicing modulate the function of Pax-2 isoforms? Due to the limited number of Pax-2 variants described so far, the consequences of Pax-2 splicing on protein function are poorly understood. Following predictions can however be made. Exons 1 to 4 of vertebrate Pax-2 transcripts encode the paired domain, which is sufficient to mediate DNA binding (Epstein et al., 1994a; Fickenscher et al., 1993). Since these exons are also present in all XPax-2 isoforms, it is very likely that all will be able to bind target sequences. The 3-nucleotide deletion found in exon 2 of XPax-2(8) and XPax-2(9) might, however, modify the DNA binding characteristics as has been described recently for Pax-3 and Pax-7 (Vogan et al., 1996). The highly conserved octapeptide sequence is encoded by exon 5 and appears to act as a repressor of the activation potential (Lechner and Dressler, 1996). Interestingly, XPax-2(5), XPax-2(6) and XPax-2(9) represent naturally occurring variants lacking exon 5 and could therefore be more potent transactivators in vivo. Comparable transactivation properties were reported for the two Pax-2 isoforms which differ by containing or lacking exon 6 (Dehbi et al., 1996; Kozmik et al., 1993;
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Lechner and Dressler, 1996). We were unable to detect XPax-2 transcripts carrying sequences related to exon 6 (Fig. 10B), although such transcripts have been described in zebrafish, mouse and humans (Dressler et al., 1990; Eccles et al., 1992; Krauss et al., 1992; Sanyanusin et al., 1996). It is possible that the expression of exon 6 containing XPax-2 transcripts is developmentally regulated in Xenopus, and occurs only in advanced embryos or in adult animals which were not analyzed in this study. Alternatively, mutations inactivating correct splicing of exon 6 might have occurred during the evolution of Xenopus laevis. In the light of the apparent lack of functional significance of exon 6, such mutations might not pose a selective disadvantage for carriers of the mutations. Proof whether such mutations occurred indeed will have to await cloning of the XPax-2 genes. The role of the partial homeodomain motif, found in all class III Pax genes, has so far not been determined. XPax-2(7), XPax-2(8) and XPax-2(9) were found to lack the homeodomain encoded by exon 7 and might therefore represent useful tools to resolve this question. Finally, alternative splice events in the C-terminal domain of Pax-2 can generate isoforms which in vitro confer differential regulation of the human WT-1 promotor (McConnell et al., 1997). The transcriptional competence appears also to be regulated by a regulatory module composed of activating and inhibitory sequences located at the C-terminal end of all class III Pax proteins (Dörfler and Busslinger, 1996). For XPax-2 isoforms, the presence or absence of exon 11 represents the only splice event observed in this region. Inclusion of exon 11 would extend the transactivation domain defined by Dörfler et al. (1996) and could modulate the transactivating properties of the described regulatory module. In summary, the nine distinct Pax-2 cDNAs isolated in this study can be considered as an unique collection of variants generated by in vivo "deletion mutagenesis". Testing their DNA binding and transactivation action properties will complement previous structure-function studies carried out with in vitro mutagenized Pax-2 proteins (Dörfler and Busslinger, 1996; Lechner and Dressler, 1996).

Developmental regulation of Pax-2 splicing

For transcription factors, developmental regulation of alternative splicing is a widespread phenomenon, but the biological consequences are understood in only a very few cases (López, 1995). A number of recent studies have demonstrated that alternative spliced products of Pax-8 are not only temporally, but also spatially regulated during embryogenesis (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). wo previous studies using nuclease protection assays had failed to detect any temporal nor spatial regulation of Pax-2 splicing.
(Dressler and Douglass, 1992; Fickenscher et al., 1993). The probes used in these studies span only a limited amount of the coding sequence and were chosen with the intention to discriminate between the two only Pax-2 splice variants known at the time. Many of the limitations inherent to nuclease protection assays have been overcome by the PCR-based methodology used in this study. In particular, we are now able to display the entire spectrum of splice variants within the experimental boundaries defined by the PCR primers used. We therefore reinvestigated comprehensively the question of developmental regulation of Pax-2 splicing by analysis of whole embryos, explants of enriched pronephric tissues and in vitro generated tissues. Our studies on whole embryos clearly established that alternative splicing of XPax-2 transcripts is under temporal control during embryogenesis (Fig. 10). A limited number of XPax-2 transcripts represented by XPax-2(7), XPax-2(8) and XPax-2(9) were found to be expressed already as maternal transcripts in the pre-gastrula embryo. These maternal isoforms are characterized by the lack exons 6, 7, 8, and 9. They however still contain the minimal structural elements to mediate DNA binding and transactivation properties. The functional significance of maternal Pax-2 transcripts is unclear, especially since Pax-2 function is not necessary for gastrulation (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995; Torres et al., 1996). The completion of gastrulation is marked by a sudden burst of XPax-2 gene expression. At this time point, the complete range of XPax-2 splice variants can already be detected (Fig. 10). In situ hybridization identifies the future midbrain-hindbrain boundary as the only source of XPax-2 gene expression at this developmental stage (Fig. 7A, B). This observation suggests that a single tissue can generate the full spectrum of Pax-2 isoforms. We then focused our attention to the developing pronephric kidney. Expiants enriched for pronephroi as well as in vitro generated pronephric tissues were examined to determine the extent of XPax-2 isoform expression in this organ (Fig. 11, 12). The results presented in this study clearly demonstrate that pronephric kidneys express the complete set of XPax-2 isoforms and that there is little variation in the relative abundance of transcripts during pronephric kidney organogenesis. A similar apparent lack of tissue-specific regulation of alternative splicing is also seen with Pax-3, Pax-6 and Pax-7 (Epstein et al., 1994b; Vogan et al., 1996). The analysis was however limited to single splice events as in previous studies addressing Pax-2 splicing (Dahl et al., 1997; Dressler and Douglass, 1992; Fickenscher et al., 1993). In the present study, we demonstrate now for pronephroi that the absence of tissue-specificity extents to all splice events for Pax-2. It is therefore rather unlikely that certain
isoforms of Pax-2 could have tissue- or organ-specific functions. Our findings rather suggest that alternative splicing may simply serve to increase the functional diversity of Pax-2 genes during embryogenesis. Further characterization of the Pax-2 splice variants will now allow us to design experiments in which the expression levels of different isoforms in *Xenopus* embryos and in growth factor-treated animal cap cultures will be altered. Ultimately, we hope to achieve a better understanding of how the different Pax-2 isoforms function in establishing and controlling the inductive cascades of events necessary for kidney organogenesis.
5.1.5. Materials and Methods

Growth factors and retinoic acid

Recombinant Xenopus basic FGF (bFGF) was prepared from E. coli strain XF140 using a T7 expression system essentially as described (Kimelman et al., 1988). An additional ion-exchange chromatography step was added to the protocol. In brief, the cleared bacterial lysate was adjusted to 0.1 M sodium phosphate, pH 6.0, and loaded onto a 80-ml CM Sephadex C25 column (Pharmacia Biotech). The column was washed with 0.1 M sodium phosphate, pH 6.0, and then with 0.1 M sodium phosphate, 0.1 M NaCl, pH 6.0. The bound protein was eluted with 0.1 M sodium phosphate, 0.4 M NaCl, pH 6.0. Peak fractions were collected, pooled, and applied to a 5-ml HiTrap Heparin column (Pharmacia Biotech). The column was washed first with 0.6 M NaCl, 10 mM Hepes, pH 7.2 and then with 1 M NaCl, 10 mM Hepes, pH 7.2. The bound protein was eluted with 2 M NaCl, 10 mM Hepes, pH 7.2. The eluates were concentrated to 10 mg/ml and the buffer was exchanged with 10 M Tris-HCl, pH 7.0 using Centriprep-10 concentration devices (Millipore). The purity of each preparation was assessed by SDS-polyacrylamide electrophoresis. Only preparations consisting of a single 17 kDa band were used. For animal cap experiments, purified Xenopus bFGF preparation were diluted with 0.5x MMR (1x MMR: 100 mM NaCl; 2 mM KCl; 2 mM CaCl2; 1 mM MgSO4; 1 mM EDTA; 5 mM Hepes, pH 7.4) containing 1 mg/ml BSA to a concentration of 10 μg/ml and stored at -80°C. Each preparation was also tested in animal cap assays for bioactivity (Kimelman et al., 1988). Xenopus bFGF was usually used at 100 ng/ml.

Recombinant human activin A was kindly provided by Kristin Verschueren and Danny Huylebroeck (Laboratory of Molecular Biology, University of Leuven, Leuven, Belgium). Activin A was dissolved in 0.5x MMR containing 1 mg/ml BSA to a concentration of 1 μg/ml and stored at -80°C. The bioactivity of the activin A preparation was tested by serial dilution using the animal cap assay. A working concentration of 5-10 ng/ml was found to give a robust induction as determined by massive elongation of the animal cap explants.

All-trans retinoic acid (Sigma, R-2625) was dissolved in dimethylsulfoxide (DMSO) at 10 mM under dim light. Aliquots were stored at -80°C. Retinoic acid was used at a concentration of 10 μM (Moriya et al., 1993).
**Embryo collection and microdissections**

Pigmented *Xenopus laevis* frogs were purchased from African Reptile Park (Tokai, South Africa), and albinos from Dr. Charles Thiébaud (Station de zoologie expérimentale, Université de Genève, Chêne-Bougeries, Switzerland). *Xenopus* embryos were obtained by *in vitro* fertilization as previously described (Brändli and Kirschner, 1995; Newport and Kirschner, 1982). Embryos were staged according to Nieuwkoop and Faber (1956).

All embryo dissections were performed in 1x MMR. Embryos were raised to stage 24 and 36 for dissection of heads and pronephric explants. Embryos were anesthetized in 1x MMR containing 0.1% tricaine (ethyl-3-aminobenzoate methansulfonic acid; Sigma, A-5040). Dissections were done using a microdissecting knife and watch-makers forceps (Fine Science Tools). First, the heads were cut immediately posterior to the visceral arches. Then, explants enriched for pronephroi ('pronephric explants') were obtained by placing a horizontal cut just below the somites to separate dorsal structures (notochord, somites, and neural tube) from ventrolateral tissues (pronephroi, ventral mesoderm, endoderm). Heads and pronephric explants were frozen in liquid nitrogen for later isolation of RNA. See also Fig. 11A, for an outline of the dissection strategy.

**Animal cap assays**

Animal caps were isolated from stage 8-9 embryos using watch-makers forceps (Dumont No. 5; Fine Science Tools), eye-brow knives and hair loops. The area of dissection was limited to the upper portion of the animal hemisphere, corresponding to roughly one fourth of the embryo. Care was taken to avoid any marginal zone cells, or large, vegetal cells. Explants were cultured in 0.5x MMR containing 1 mg/ml bovine serum albumin (Sigma, A-3350). Purified growth factors (*Xenopus* bFGF or human activin A) and retinoic acid were added at the desired concentrations. Animal caps were incubated in the dark at 22°C for 12 hrs, after which they were rinsed and transferred to 0.5x MMR/BSA. Animal caps were cultured until control embryos reached stage 28. For further analysis, animal caps were either frozen in liquid nitrogen for later isolation of RNA or fixed for whole mount *in situ* hybridization.

**Isolation of XPax-2 cDNAs by library screening**

Partial DNA sequences of *Xenopus* Pax-2 were isolated by the reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers.
The following degenerate primer pair was used (see pp. 72/3 for sequences): XP-1 coding for the target peptide RQRIVE; XP-2 coding for G(V/I)CDN(D/E). EcoR I and Xba I restriction sites were added to the sense and antisense primers, respectively, for convenient ligation. Complementary DNAs (cDNAs) were synthesized from 5 μg of total RNA isolated from stage 21, 24 and 30 Xenopus embryos, respectively, using 500 units of Molony murine leukemia virus reverse transcriptase (Superscript II, GIBCO BRL) and 200 pmol of degenerate primer XP-2 in a total volume of 50 μl. One-twentieth of the cDNA preparation was used as a template for amplification of Pax sequences. PCR was performed in a final volume of 50 μl containing 10 mM Tris pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTPs, 100 pmol of each primer (XP-1 and XP-2), and 2.5 units of Taq DNA polymerase (Perkin-Elmer). The amplification parameters were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 35 cycles followed by 10 min at 72°C. One-third of the amplification products were size fractionated on a 4% NuSieve 3:1 agarose (FMC BioProducts) gel. Amplification products of the expected size, ~280 bp, were excised, and purified using GENECLEAN SPIN kit (BIO 101 Inc.). The recovered amplification products were digested with EcoR I and Xba I, repurified and cloned into pBluescript II SK+ vectors (Stratagene). Competent E. coli DH5α bacteria (MAX efficiency, GIBCO BRL) were used for transformation. The three cDNAs libraries (st. 21, 24, and 30) were screened for clones with sequences homologous to Pax-2. Altogether 88 cDNA clones were analyzed by restriction fragment mapping and DNA sequencing as described (Brändli and Kirschner, 1995). DNA sequences were determined from alkaline lysis miniprep DNA using the dideoxy chain termination method (Sequenase version 2.0, Amersham).

Amplification products encoding XPax-2a and XPax-2b were used to screen a Xenopus stage 28-30 head cDNA library (Hemmati-Brivanlou et al., 1991) ligated into an excisable λZAPII vector (kindly provided by Richard Harland, University of California at Berkeley). Fragments were radiolabeled with [³²P]dCTP (Amersham) by random priming (Oligolabelling Kit, Pharmacia Biotech), and purified on NUCTRAP push columns (Stratagene). Library screening was carried out under conditions of high stringency (Maniatis et al., 1989). Positive clones were purified, and converted to Bluescript plasmids using the Rapid Excision Kit (Stratagene) according to the manufacturer’s recommendations. Plasmids were analyzed by restriction fragment mapping and DNA sequencing. Three cDNA classes were identified, and the longest cDNAs [2.0 kb for XPax-2a(1), 3.0 kb for XPax-2a(3), and 2.7 kb for XPax-
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2b(2)] were sequenced. The EMBL Nucleotide Sequence Database accession numbers can be found in the next section.

Isolation of XPax-2 cDNAs by PCR amplification

Sequences common to both XPax-2 genes were chosen to synthesize oligonucleotide primers with the aim of amplifying by PCR the entire open reading frame of XPax-2 cDNAs. Primer XP-26 corresponds to nucleotides in exon 1 starting at the first translational start site. Primer XP-15 is complimentary to sequences in the 3'-untranslated region of exon 13. Sequences of primers are listed on pp. 72/3. Nucleotide sequences encoding EcoR I and Xba I restriction sites were added to the 5'-ends of XP-26 and XP-15, respectively. *Xenopus* explants enriched for pronephroi were microdissected from stage 24 embryos (see 4.2. and Fig. 11A) and used for isolation of total RNA (see 4.6.). First strand cDNA was generated essential as described (see 4.4.) using 0.2 pmol of the antisense primer XP-15 and 10 μg of total RNA. PCR amplification was carried out with the Expand High Fidelity PCR system (Boehringer Mannheim) using one-fortieth of the cDNA preparation as a template. The resulting amplification products were separated by electrophoresis on a 1% agarose gel. A section of the gel containing amplification products ranging between 0.7 and 1.8 kb in size was isolated, and cut into eight slices containing products of increasing size. For each slice, DNA was purified, enriched by a second round of PCR amplification and cloned into the pBluescript II SK (+). From the eight PCR-generated cDNA libraries, a total of 140 cDNA clones were selected and further analyzed by PCR. PCR reactions using different sets of primer pairs were performed to determine the exon composition of each cDNA. Clones representing novel splice variants of XPax-2 were confirmed by sequencing the entire cDNA.

The nucleotide sequences of all XPax-2 cDNAs were submitted to the EMBL Nucleotide Sequence Database, and the accession numbers are as follows: Y10119 for XPax-2a(1), Y10120 for XPax-2b(2), Y10121 for XPax-2a(3), Y10122 for XPax-2a(4), Y10123 for XPax-2a(5), AJ000666 for XPax-2(6), AJ000667 for XPax-2a(7), AJ000668 for XPax-2b(8), and AJ000669 for XPax-2(9).

RNA isolation

Total RNA was isolated from unfertilized eggs, embryos and explants using the guanidinium-isothiocyanate method (TRIzol Reagent; Gibco BRL).
The RNA was precipitated with 1/10 vol. of 0.5 M sodium acetate, pH 5.2 and 2 vol. of 100% ethanol. Precipitated RNA was washed with 70% ethanol and resuspended in an appropriate volume of water. RNA concentrations were determined spectrophotometrically.

RT-PCR and Southern blot analysis

For amplification of cDNAs encoding alternatively spliced Pax-2 transcripts, total RNA was extracted from unfertilized eggs, embryos of different developmental stages, and from tissue samples (heads, pronephric explants, and animal caps) as described above. A mixture of XPax-2 antisense primers (XP-11, XP-24, XP-31, XP-63; 0.2 pmol each) was used to synthesize first-strand cDNAs from 10 μg of total RNA as described in the above section. Furthermore, antisense primers (EF1α-2, GAPDH-2, H4-2 and ODC-2; 0.2 pmol each) complementary to Xenopus elongation factor 1α (EF1α), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), histone H4 and ornithine decarboxylase (ODC), mRNAs were included in the cDNA synthesis reaction to control for equal mRNA concentrations. One-fortieth of the cDNA preparation was used as template for subsequent amplification by PCR. Where cloned XPax-2 cDNAs were used as templates for PCR, purified plasmids were added as templates at 1 ng per 50-μl reaction. Each primer was added at 50 pmol. The amplification parameters were 94°C for 1 min, 48°C for 40 sec, and 72°C for 35 cycles followed by 10 min at 72°C. One-third of the amplification product was size fractionated on either a 4% or 6% NuSieve 3:1 agarose gel.

Agarose gels were subjected to Southern blotting and hybridized with radiolabeled XPax-2-specific probes for unequivocal identification of amplification products encoding XPax-2 sequences. In brief, amplification products were capillary blotted onto nylon membranes (HYBOND-N+, Amersham) in 0.4 N NaOH and crosslinked to the membrane by UV-irradiation (UV Stratalinker 1800, Stratagene). The filters were hybridized for 18 h in hybridization buffer (5x SSPE, 5x Denhardt's solution, 0.5% SDS, 20 μg/ml denatured fragmented Salmon sperm DNA) at 65°C with a radiolabeled probe. After hybridization, the filters were washed twice for 10 min in 2x SSPE, 0.1% (w/v) SDS at room temperature and once for 10 min in 0.2x SSPE, 0.1% (w/v) SDS at 65°C.

DNA for the synthesis of radiolabeled probes to detect splicing in the paired domain region (probe 1; primers: XP-25 and XP-31) or the octapeptide region (probe 2; primers: XP-59 and XP-11) were generated by PCR with XPax-2a(1) cDNA (1 ng per 50-μl reaction) as template DNA. The purified
amplification products were radiolabeled by random priming as described (see 4.4.). In all other cases, probe 3 directed against both allelic forms of exon 10 was used for hybridization. Radiolabeled probe 3 DNA was generated by PCR using the primers XP-62 and XP-63 and XPax-2a (1) and XPax-2b(2) cDNAs as templates. PCR was performed in a final volume of 30 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 16 µM of dATPs, dGTPs and dTTPs, 5 pmol of [³²P]dCTP, 1 pmol of each primer (XP-62 and XP-63), and 1 unit of Taq DNA polymerase (Perkin-Elmer). The amplification parameters were 94°C for 45 sec, 48°C for 2 min, and 72°C for 1 min for 10 cycles. The radiolabeled probe 3 was purified using a G50 Sephadex column (Boehringer Mannheim) according to the manufacturer’s recommendations.

Amplification products were verified by DNA sequencing as follows. After separation by agarose gel electrophoresis, bands were excised from the gel, purified and subcloned into the PCR2.1 vector (Original TA Cloning Kit, Invitrogen) according to the manufacturer’s recommendations.

Primers

The following primers were used for cDNA synthesis and PCR amplification:

EF1α1: 5′-CAG ATT GGT GCT GGA TAT GC-3′ (sense primer)
EF1α2: 5′- ACT GCC TTTG ATG ACG CCT AG -3′ (antisense primer)
GAPDH-1: 5′-ATT CCC GCC TCA ACT GT-GG-3′ (sense primer)
GAPDH-2: 5′-AGG ATG GGC GAG TCT GC-3′ (antisense primer)
H4-1: 5′-ATA ACA TCC AGG GCA TCA CC-3′ (sense primer)
H4-2: 5′-ACA TCC ATA GCG GTG AGC GT-3′ (antisense primer)
ODC-1: 5′-GTG AAT GAT GGA GTG TAT GGA TC-3′ (sense primer)
ODC-2: 5′-TCC ATT CCG CTC TCC TGA GCA C-3′ (antisense primer)
XP-1: 5′-(C/A)GI CA(A/G) (C/A)GI AT(A/C/T) GTI GA-3′ (sense primer)
XP-2: 5′-TC(A/G) TT(A/G) TC(A/G) CAI A(C/T)I CC-3′ (antisense primer)
XP-9: 5′-GAC CTA GGA AGC AAT GT-3′ (sense primer)
XP-11: 5′-AAA GTTTCA ACACTG CT-3′ (antisense primer)
XP-15: 5′-GGT ACA ATATGG AGG CC-3′ (antisense primer)
XP-24: 5′-GCC CCTGGATGT GGCAC-3′ (sense primer)
XP-25: 5′-GGA TAT GCACTG CAA GG-3′ (sense primer)
XP-26: 5′-ATG GATATG CAC TGC AAG G-3′ (sense primer)
XP-31: 5′-CCA GGTACC ATT CCA GC-3′ (antisense primer)
Whole mount in situ hybridization and probe synthesis

Whole mount in situ hybridization was performed using digoxigenin-labeled RNA probes (Harland, 1991). The standard protocol was modified by replacing methanol with ethanol, and by omitting the RNase digestion step. Fixed animal cap explants were bleached in 70% ethanol/10% H2O2 prior to in situ hybridization.

The following plasmids were constructed for probe synthesis: (a) plasmid Bs4A.3, containing the entire coding sequence (nucleotides 19-1203) of XPax-2a(1); (b) plasmid Bs4B.4, containing the C-terminal domain (nucleotides 799-1203) of XPax-2a(1); (c) plasmid Bs10B.4’, containing the C-terminal domain (nucleotides 900-1403) of XPax-2a(3); (d) plasmid Bs7B.5, containing the C-terminal domain (nucleotides 850-1464) of XPax-2b(2); (e) pBs7C.1, containing exon 9 (nucleotides 988-1197) of XPax-2b(2); and (f) pBs10C.1, containing exon 11 (nucleotides 1140-1238) of XPax-2a(3). The plasmids were generated using PCR-based subcloning procedures. The PCR primers were designed with EcoR I (for sense primers) and Xba I (for antisense primers) restriction sites for convenient ligation. Amplification products were ligated into Bluescript II SK+ vector.

Digoxigenin-labeled antisense RNA probes were synthesized with T3 RNA polymerase (MBI Fermentas) from EcoR I linearized plasmids. For control purposes, sense probes were synthesized with T7 RNA polymerase from Xba I linearized plasmids. The in vitro synthesized probes had the following approximate sizes: 1.2 kb for plasmid Bs4A.3; 0.4 kb for plasmid Bs4B.4; 0.5 kb for plasmid Bs10B.4’; 0.6 kb for plasmid Bs7B.5; 0.2 kb for plasmid Bs7C.1; and 0.1 kb for plasmid Bs10C.1.

Stained embryos were visualized and photographed using a Zeiss STEMI 2000-C stereoscopic microscope equipped with a Zeiss MC80 camera using either Kodak Ektachrome 64T or Fujichrome 64T professional slide film. Color slides were scanned using a slide scanner (Polaroid SprintScan 35), and processed using Adobe Photoshop 3.0 and Canvas 3.0 software.

Tissue sectioning

Embryos stained in whole mount were fixed for 1-2 h in MEMFA (Harland, 1991), washed with PBS and embedded in 3% low-melting agarose (SeaPlaque, FMC BioProducts). 50-100 μm sections were cut with a vibrating blade microtome (Leica VT1000M). Photographs were taken and processed exactly as described above.
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5.2. *Xenopus* Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages

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5.2.1. Abstract

Pax genes are a family of transcription factors playing fundamental roles during organogenesis. We have recently demonstrated the expression of Pax-2 during *Xenopus* embryogenesis [Heller, N, Brändli, AW (1997) Mech Dev 69, 83-104]. Here we report now the cloning and characterization of *Xenopus* Pax-5 and Pax-8, two orthologues of the Pax-2/5/8 gene family. Molecular phylogenetic analysis indicates that the amphibian Pax-2/5/8 genes are close relatives of their mammalian counterparts and that all vertebrate Pax-2/5/8 genes are derived from a single ancestral gene. *Xenopus* Pax-2/5/8 genes are expressed in spatially and temporally overlapping patterns during development of at least seven distinct tissues. Most strikingly, *Xenopus* Pax-8 was identified as the earliest marker of the prospective otic placode and of the intermediate mesoderm, indicating that Pax-8 may play a central role in auditory and excretory system development. Comparison of the expression patterns of fish, amphibian and mammalian Pax-2/5/8 genes revealed that the tissue-specificity of Pax-2/5/8 gene family expression is overall evolutionary conserved. The expression domains of individual orthologues can however vary in a species-specific manner. For example, the thyroid glands of mammals express Pax-8, while in *Xenopus* Pax-2 is expressed instead. Our findings indicate that differential silencing of Pax-2/5/8 gene expression may have occurred after the different classes of vertebrates began to evolve separately.
5.2.2. Introduction

The genesis of organs during embryonic development is regulated by inductive interactions and a complex process of activation and deactivation of genes controlling cellular fate and differentiation. Many of these developmental control genes code for transcription factors and are able to initiate expression of a cascade of subordinate genes necessary for proper organ development. It is therefore not surprising to find that mutations of these genes underlie a number of congenital syndromes (Engelkamp and van Heyningen, 1996). Pax genes constitute an important family of transcription factors playing a pivotal role as regulators of embryonic development (Dahl et al., 1997; Mansouri et al., 1996). The defining characteristics of this family is the presence of a 128-amino acid long paired domain encoding a unique DNA-binding motif (Chalepakis et al., 1991; Treisman et al., 1991). In addition, many of these genes contain a complete or truncated DNA-binding homeodomain and/or an octapeptide motif. The paired box was first identified in Drosophila, and homologs have been found in a variety of vertebrates and invertebrates. Nine Pax genes have been identified in human and mouse. Sequence similarity and gene structure indicate that the mammalian Pax gene family consists of four well-defined groups: group I (Pax-1/9), II (Pax-2/5/8), III (Pax-3/7), and IV (Pax-4/6) (Balczarek et al., 1997; Walther et al., 1991). During vertebrate development, most Pax genes are expressed in complex patterns within the central nervous system (Chalepakis et al., 1993; Mansouri et al., 1996).

Members of the Pax-2/5/8 gene family are also prominently expressed outside of the central nervous system implicating functions during development of the kidney (Pax-2 and Pax-8), the visceral arches (Pax-2), the thyroid gland (Pax-8), and B lymphocytes (Pax-5) (Adams et al., 1992; Asano and Gruss, 1992; Dressler et al., 1990; Heller and Brändli, 1997; Plachov et al., 1990). Pax-2/5/8 gene family members have recently been associated with human disease syndromes and specific developmental defects in mouse mutants. Patients with renal coloboma syndrome are heterozygous for PAX-2 mutations and have optic nerve colobomas, renal hypoplasia, and occasional hearing defects (Sanyanusin et al., 1995b). Natural and targeted mouse mutants demonstrate that Pax-2 is involved in multiple steps during urogenital development as demonstrated by the absence of kidneys, ureters and genital tracts (Favor et al., 1996; Torres et al., 1995). Similar to humans, mutant animals exhibit also defects in the eye (bilateral coloboma) and the inner ear (lack of cochlear outgrowth) (Favor et al., 1996; Torres et al., 1996). Expression of Pax-2 and Pax-5 overlaps spatially and temporally at the midbrain-hindbrain boundary.
Results

(MHB) suggesting redundant functions. Indeed, homozygous mice deficient in either Pax-2 or Pax-5 show no gross defects in derivatives of the MHB (Schwarz et al., 1997; Urbánek et al., 1994). Analysis of double Pax-2/Pax-5 mutants reveals however a deletion of the cerebellum and alterations of the tectum and tegmentum (Schwarz et al., 1997). Early B-lymphopoiesis is critically dependent on Pax-5 gene function. In the fetal liver, Pax-5 is required for differentiation of the earliest B-lineage committed precursor cells (Nutt et al., 1997; Urbánek et al., 1994). Deregulation of PAX-5 in humans, elicited by a chromosomal translocation juxtaposing an IgH enhancer upstream of the PAX-5 locus, may contribute to the pathogenesis of a subset of non-Hodgkin's lymphomas (Busslinger et al., 1996). Probably due to partially redundant functions provided by Pax-2 and Pax-5, Pax-8 mutant mice do not show obvious defects of the spinal cord, the MHB and the kidneys. Pax-8 is however required in the mouse for the formation of the follicular cells in the thyroid gland (Mansouri et al., 1998), and in humans heterozygosity for PAX-8 is associated with congenital hypothyroidism (Macchia et al., 1998). Together, these observation underscore the important role of the Pax-2/5/8 gene family in the control of various organogenesis processes.

Malformations of the kidneys occur sporadically, but on occasion they can also be familial. Affected individuals may have unilateral or bilateral disease which may take the form of either renal hypoplasia and/or agenesis. Renal malformations are believed to occur in 1:3,000 to 1:10,000 newborns and are the most commonest causes of chronic renal failure in the first years of life (Woolf, 1995). Early diagnosis and therapy therefore is absolutely critical. Recent evidence suggests that mutations of developmental control genes, such as PAX-2, SALL-1 and EYA-1, may cause familial renal malformations (Abdelhak et al., 1997; Kohlhase et al., 1998; Sanyanusin et al., 1995b). In order to understand the molecular basis of malformations of the kidney, it is therefore essential to decipher the other members in the cascades of molecular events underlying kidney organogenesis.

Early steps of kidney organogenesis, in particular the processes leading to the commitment of cells to the nephric fate and subsequent formation of the pronephric kidney, are difficult to study in the mammalian embryo, but can be more readily assessed in the amphibian embryo (Vize et al., 1997). We have recently reported the cloning of *Xenopus* Pax-2, and showed that expression of this gene is associated with the onset of pronephric kidney morphogenesis (Heller and Brändli, 1997). Here we describe now the cloning, molecular phylogeny and developmental expression of the *Xenopus* Pax-5 and Pax-8.
genes. We found that expression of Pax-8 is associated with the prospective otic placode and the intermediate mesoderm. Expression is initiated at the same time point when otic fate and pronephric tubules become specified in the early neurula embryo. To the best of our knowledge, Pax-8 is the earliest gene known to be expressed in these tissues and may therefore be on top of molecular cascades controlling inner ear development and early kidney organogenesis.
5.2.3. Results

**Cloning of Xenopus Pax-5 and Pax-8 cDNAs**

A polymerase chain reaction (PCR) fragment encoding a peptide closely resembling mammalian Pax-5 had previously been isolated (Heller and Brändli, 1997). This fragment was used for screening of a Xenopus cDNA library, and a partial cDNA encoding the N-terminal 180 amino acids of a paired box protein was isolated. In mammals, the Pax-5 gene is transcribed from two distinct promoters, resulting in alternative splicing of different 5' end exons, 1A or 1B, to the common coding sequences of exons 2-10. Transcripts containing exon 1A are expressed predominantly in B-lymphocytes, while exon 1B containing transcripts are found in all Pax-5 expression domains (Busslinger et al., 1996). Sequence comparisons showed that the N-terminus of the Xenopus protein shared 13 out 14 amino acids with exon 1B of mouse and human Pax-5 (Fig. 13).

The missing 3' Xenopus Pax-5 sequences were isolated by rapid amplification of cDNA ends (RACE)-PCR. Subsequently, reverse transcription (RT)-PCR was performed to obtain cDNAs encoding the complete open reading frame (ORF) of Xenopus Pax-5. Sequence analysis led to the identification of five different classes of Xenopus Pax-5 cDNAs, Pax-5(1) to Pax-5(5). The observed cDNA variants are probably the result of alternative splicing events, as previously seen with mouse and human Pax-5 (Busslinger et al., 1996; Zwollo et al., 1997). Pax-5(1) encodes a protein that is collinear mammalian Pax-5 proteins and lacks the exon 9.1 found in zebrafish Pax-5 (Fig. 13). For Pax-5(2), usage of an alternative splice site located within intron 6 results in 108 additional nucleotides being retained between exon 6 and 7. The original reading frame remains however unaltered (Fig. 14A). Pax-5(3) encodes a truncated protein, which is generated by activation of an alternative splice site located in exon 2. This results in a deletion of 30 nucleotides and creates a premature stop codon at the junction site (Fig. 14B). Pax-5(4) and Pax-5(5) lack exon 2 or exon 7, respectively, resulting in truncated proteins (Fig. 14C, D). Nucleotide sequence comparisons suggest that all Pax-5 cDNAs are derived from transcripts of a single Xenopus Pax-5 gene (not shown).
Results

Fig. 13. Sequence comparison of vertebrate Pax-5 proteins. Xenopus Pax-5(1) splice variant has been aligned with various vertebrate Pax-5 proteins. Human and mouse Pax-5 are shown with the alternatively spliced exon 1B (Busslinger et al., 1996). GenBank accession numbers to the human, mouse and zebrafish Pax-5 sequences are listed in Fig. 16. The paired domain and the partial homeodomain have been highlighted by shading. The octapeptide domain has been underlined. Amino acids shared by all four proteins are boxed. Dashes indicate gaps that were introduced for optimal sequence alignment. Exons are numbered according to Pfeffer et al. (1998).
PAX GENES AS REGULATORS OF EARLY XENOPUS EMBRYOGENESIS

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- **β-galactosidase staining**
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- **Identification of the regulatory elements conferring pronephric expression of the Pax-2 and Pax-8 genes using transgenic frogs**
- **Identification of downstream targets of Pax-2 and Pax-8 in the molecular cascade controlling pronephric kidney development**
- **Determination of the DNA binding and transactivation activities of Pax-2 isoforms**

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### 9. Curriculum Vitae
1. Zusammenfassung

Die Entwicklung der Exkretionsorgane im Wirbeltierembryo verläuft in einer räumlich und zeitlich präzise geregelter Abfolge, die durch die Bildung von drei verschiedenen Nieren, der pronephrischen (Vorniere), der mesonephrischen (Urniere) und der metanephrischen Niere (Nachniere) charakterisiert ist. An der Vorniere lassen sich zwei charakteristische Strukturen unterscheiden, die sogenannten Nierentubuli und der Vornierengang (pronephrische oder Wolff 'sche Gang). Der pronephrische Gang nimmt in der Nierenentwicklung eine zentrale Bedeutung ein, da er unter anderem die Ausbildung der mesonephrischen und metanephrischen Niere einleitet. Während die pronephrische Niere der höheren Vertebraten nur eine transiente Struktur darstellt, entwickelt sie sich in Embryonen der Fische und Amphibien zu einem voll funktionsfähigen Organ. Froschembryonen sind daher besonders gut geeignet, um die molekularen Mechanismen der frühen Nierenbildung zu untersuchen.


2. Summary

Development of the excretory system occurs in a series of distinct steps during vertebrate embryogenesis. Three distinct excretory organs, the pronephric, mesonephric, and metanephric kidney, are formed in precise spatial and temporal sequence. The pronephric kidney consists of a tubular system and the pronephric duct which is essential for later steps of vertebrate kidney development. In higher vertebrates, the pronephric kidney is a transient structure. In embryos of fish and amphibia, however, the pronephric excretory system becomes fully functional. Embryos of the frog *Xenopus laevis* represent therefore an ideal model to elucidate the molecular mechanisms underlying early kidney organogenesis.

Pax genes are important developmental control genes as illustrated by mutations in zebrafish, mice and human patients. Among the nine members of the vertebrate Pax gene family, the Pax-2/5/8 genes comprise a separate group which is of particular interest with respect to kidney development. Analysis of Pax-2 deficient mice revealed that Pax-2 might represent a primary regulator of early kidney organogenesis. Furthermore, patients carrying mutations in the Pax-2 gene suffer from renal-coloboma syndrome, an autosomal dominant disease leading to renal insufficiency.

A reverse transcriptase-polymerase chain reaction (RT-PCR) strategy was used to isolate cDNAs encoding all three *Xenopus* Pax-2/5/8 orthologues. Molecular phylogenetic analysis indicates that the amphibian Pax-2/5/8 genes are close relatives of their mammalian counterparts and that all vertebrate Pax-2/5/8 genes are derived from a single ancestral gene. *Xenopus* Pax-2/5/8 genes are expressed in spatially and temporally overlapping patterns during the development of at least seven distinct tissues. Expression of Pax-2 was confined to the nervous system, sensory organs, the visceral arches, and the developing excretory system. In the developing pronephric kidney, Pax-2 expression was initiated at the onset of morphogenesis and detectable in all epithelial structures of the organ. Pax-5 was expressed in the developing midbrain-hindbrain boundary (MHB) and in the otic vesicle, but not in the pronephric kidney. Finally, Pax-8 transcripts were detected in the prospective otic region giving later rise to the otic vesicle and in the developing pronephric kidney. Most strikingly, Pax-8 was identified as the earliest marker of the intermediate mesoderm and thus of the pronephric lineage.

Alternative splicing of Pax gene transcripts is frequently observed, and multiple splice products were also detected for *Xenopus* Pax-2/5/8 transcripts.
The screening of cDNA libraries led to the identification of nine different *Xenopus* Pax-2 cDNAs of which seven encoded novel Pax-2 isoforms. All Pax-2 isoforms retained their DNA-binding domains, but differed significantly in their C-termini. The regulation of Pax-2 splicing was assessed by analyzing different embryonic tissues and whole *Xenopus* embryos. Alternative splicing of Pax-2 transcripts was under temporal regulation during *Xenopus* embryogenesis. Analysis of dissected embryonic heads and pronephric kidneys, as well as animal cap explant cultures could however not provide evidence for tissue-specific splicing.

Several *in vitro* studies have reported differences in the biochemical properties of Pax gene splice variants. Here, the role of Pax-2 isoforms in pronephric kidney organogenesis and MHB development was assessed by injecting *Xenopus* embryos with RNAs encoding single Pax-2 splice variants. Overexpression of Pax-2 proteins led to an enlargement of the expression domains of early and late pronephric marker genes. Pax-2 appears therefore to act at an early step in the molecular cascade controlling kidney development. Ectopic expression of Pax-2 resulted also in enlarged expression domains of MHB markers. Furthermore, ectopic cells expressing MHB marker genes were detected in vicinity to the MHB. The phenotypes obtained in Pax-2 injected embryos suggest a role for Pax-2 in cell fate determination and/or proliferation control during kidney organogenesis and MHB development. Most strikingly, functional differences were observed between different Pax-2 isoforms. These findings suggest that alternative splicing serves to increase the functional diversity of Pax-2 genes during embryogenesis.
3. Introduction

3.1. Principles of embryonic development

Complex, multicellular organisms are a consequence of tightly controlled and coordinated processes during embryogenesis. In the course of embryonic development, undifferentiated cells mature into differentiated cell populations that form tissues and organs with specific functions. Both, embryonic patterning and organogenesis comprise a series of coordinated events that are initiated by inductive signals emitted from one cell population determining the developmental fate of other cells. Once cells become committed to a particular developmental fate, organogenesis and tissue patterning proceed through morphogenesis and progressive differentiation leading to the formation of organs and structures (Gilbert, 1997). The factors directing these complex processes comprise a wide variety of proteins such as secreted factors, membrane-bound receptors, and transcription factors (Kessel and Gruss, 1990).

Although all cells contain the same genetic information, the correct spatial and temporal fate of the different cell populations during embryogenesis is ensured by the expression of an unique subset of the total number of available genes in each cell type. The selected expression of developmental genes requires a reliable, specific, and tight control. Consequently, transcription factors as the most direct regulators of gene transcription play an important role in the development of a multicellular organism (Calkhoven and Ab, 1996). By classical definition, a transcription factor is a protein that binds to DNA in a sequence-specific manner and interacts with other components of the transcriptional machinery to enhance or inhibit transcription of specific target genes (Triezenberg, 1995). Therefore, these nuclear proteins ensure the correct timing and proper localization of gene expression by activating and deactivating corresponding genes. In concordance with their multiple and different tasks in developmental processes and in adult homeostasis, a wide variety of transcription factors does exist. Their ability of specific spatial interaction with DNA is based on various sets of DNA binding motifs which may include homeodomains, paired domains, basic helix-loop-helix domains, basic leucine zipper domains, zinc finger domains, POU domains, winged helix-turn-helix domains, high mobility group (HMG) domains or ETS-domains (Nelson, 1995).
3.2. The Pax gene family

Pax genes have been identified as a family of important developmental control genes involved in the formation of various structures and organs in the embryo (Dahl et al., 1997; Mansouri et al., 1996). Pax proteins constitute a small class of transcription factors that harbor a highly conserved DNA-binding motif of 128 amino acids, termed the paired domain, located very close to the amino terminus (Chalepakis et al., 1991; Treisman et al., 1991). Some Pax proteins also contain a complete or truncated paired-type homeodomain, whereby only a complete homeodomain represents a second DNA-binding motif (Adams et al., 1992). In addition, most Pax proteins harbor a conserved stretch of eight amino acids, the octapeptide, of unknown function. Pax proteins diverge mainly in the extreme C-terminus, which constitutes a proline/serine/threonine-rich region and contains sequence motifs mediating transactivation (Chalepakis et al., 1994; Czerny and Busslinger, 1995; Fickenscher et al., 1993).

The paired box motif is found in species of vertebrates and invertebrates indicating its high conservation during evolution. Originally, the paired domain was identified in a class of Drosophila segmentation genes that includes paired, gooseberry-proximal, gooseberry-distal, Pox meso, and Pox neuro (Baumgartner et al., 1987; Bopp et al., 1986). These genes are considered to be involved in the establishment of the Drosophila body plan. In vertebrates, nine Pax genes (designated Pax-1 to Pax-9) have been identified to date (St-Onge et al., 1995). Pax genes have been found in vertebrate species from zebrafish to man. They map on different chromosomal loci and are not organized in clusters (Pilz et al., 1993; Stapleton et al., 1993). On the basis of the assembly of structural motifs, the vertebrate Pax family is classified into four paralogous groups: group I (Pax-1/9), II (Pax-3/7), III (Pax-2/5/8), and IV (Pax-4/6) (Balczarek et al., 1997; Walther et al., 1991) (Fig. 1). Within each group, the genes are characterized by a very high degree of sequence similarity with respect to the paired domains, conservation of genomic organization, and largely overlapping expression patterns. Therefore, the paralogous genes found within each class are likely to be the result of evolutionary duplication and divergence processes (Noll, 1993).
Introduction

Pax genes Basic structure

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<td>Pax-9</td>
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<td>C</td>
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<tr>
<td>Pax-6</td>
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Fig. 1. Structure of vertebrate Pax proteins. All Pax proteins contain a paired domain (PD), a DNA binding domain of 128 amino acids, located at their amino-terminal ends. Distinct subgroups of proteins are defined by the presence or absence of a paired-type homeobox (HD) and an octapeptide motif (OP). In addition to the paired domain, Pax-3, Pax-4, Pax-6, and Pax-7 have a second DNA binding domain, the paired-type homeodomain (HD). This homeodomain is truncated in Pax-2, Pax-5 and Pax-8, and is totally absent in Pax-1 and Pax-9. Furthermore, all Pax proteins, except Pax-4 and Pax-6, carry an octapeptide motif located between the paired domain and the homeobox. Figure adapted from Dahl et al. (1997).

3.3. Expression of Pax genes during vertebrate development

The expression of Pax genes during embryogenesis has been studied most comprehensively in the mouse. Each member of the Pax gene family shows spatially and temporally restricted expression patterns during embryogenesis.

All Pax genes, except Pax-1 and Pax-9, are expressed in various restricted territories of the developing nervous system implicating a crucial role in the regionalization of the neural tube and the brain (Chalepakis et al., 1993). Pax-6 is found in the telencephalon (Walther and Gruss, 1991); Pax-3 and Pax-7 in the mesencephalon (Goulding et al., 1991; Jostes et al., 1990); and Pax-2, Pax-5, and Pax-8 at the midbrain-hindbrain boundary (Asano and Gruss, 1992; Nornes et al., 1990; Plachov et al., 1990). In the spinal cord, Pax genes display restricted expression patterns along the dorsoventral axis (Chalepakis et al.,
1993). In the developing eye, Pax-2 is exclusively expressed in the optic stalk, while Pax-6 expression is localized in the eye cup (reviewed in Macdonald and Wilson, 1996).

Besides expression in the developing nervous system, several Pax genes are also expressed during development of various organs. During somitogenesis, Pax-1, -3, -7, and -9 are expressed in restricted patterns within the paraxial mesoderm. After somite formation, Pax-1 and Pax-9 expression becomes confined to the sclerotome, whereas expression of Pax-3 and Pax-7 is associated with the dermomyotome (St-Onge et al., 1995). Pax-1 is also expressed in the thymus (Wallin et al., 1996); Pax-4 and Pax-6 are found in the pancreas (Sosa-Pineda et al., 1997; Turque et al., 1994); Pax-5 is detected in early B-lymphopoiesis (Adams et al., 1992); and Pax-8 is associated with the thyroid gland (Plachov et al., 1990). Finally, Pax-2 and Pax-8 expression is observed in the developing kidney (Dressler et al., 1990; Plachov et al., 1990).

3.4. Alternative splicing of Pax genes

The complexity of Pax protein distribution and function during development is enhanced by alternative splicing. Alternative splicing is an important mechanism generating protein diversity from a single gene by selectively excising different exons encoded by that gene during RNA processing (Smith et al., 1989). Transcripts that either contain or lack particular exons may have entirely different biochemical properties at the protein level (Foulkes and Sassone-Corsi, 1992). Particularly, transcription factors are highly modular in the organization of sequence elements required for DNA binding, dimerization, ligand binding, subcellular localization, and transcriptional activation (reviewed by Mitchell and Tjian, 1989). Interestingly, isoforms with opposite (activating or repressing) functions are often generated from the same gene by modifications in the domain responsible for the activation of transcription of target genes. Transcription factor isoforms lacking the functional activator domain, while still performing an efficient and specific recognition of target DNA motifs, act as negative regulators of transcription by competing for binding to the same DNA site with the corresponding activator domain containing isoform (López, 1995).

Alternative splicing is a common phenomenon for Pax genes. Multiple splice products have been reported for nearly all members of the Pax gene family. In vitro studies revealed that alternative splicing can modulate DNA binding affinity and specificity as well as transactivation activity. For example, insertion of an additional exon in the paired domain of Pax-6 results in
modification of the DNA binding specificity suggesting that the two isoforms regulate different sets of target genes (Epstein et al., 1994b). Furthermore, alternate use of consensus 3' splice sites lead to a deletion of three nucleotides in the paired domain of Pax-3 and Pax-7 proteins generating isoforms with increased affinity to target DNA sequences (Vogan et al., 1996). For the Pax-8, at least seven splice variants have been identified that do not differ in DNA binding activity but demonstrate distinct transactivation properties. Moreover, changes in the relative abundance of different splice variants during embryogenesis indicate that alternative splicing may be developmentally regulated (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995).

### 3.5. Functions of Pax genes in embryonic development

Based on their embryonic expression patterns, Pax genes may exert multiple functions during embryogenesis. The present understanding of Pax gene function is based mainly on in vivo studies. Analysis of ectopic expression experiments in Drosophila has identified Pax-6 as a master regulator of eye development (Halder et al., 1995). Furthermore, naturally occurring and genetically engineered mutations in Drosophila, zebrafish, mouse, and man have shed light on the requirement of Pax gene function for embryonic development. These findings will be discussed below in greater detail. Many efforts have also been directed towards the identification of target genes to elucidate the role of Pax genes in the hierarchical network of gene regulation during embryogenesis (Adams et al., 1992; Epstein et al., 1994a; Epstein et al., 1996; Song et al., 1996; Zannini et al., 1992). Finally, in vitro data indicate that an important function of Pax genes during organogenesis could be the initiation of cell proliferation (Maulbecker and Gruss, 1993; Stuart et al., 1995).

### 3.6. Mutations in Pax genes

Clinical and molecular analysis of human diseases and mouse mutants caused by Pax gene mutations suggests that vertebrate Pax genes are key regulators during embryonic development of the kidney (Pax-2), the thyroid gland (Pax-8), the pancreas (Pax-4, -6), the teeth (Pax-9), the eye (Pax-2, -6), the ear (Pax-2), the nose (Pax-6, -7), the limb muscle (Pax-3), the vertebral column (Pax-1), the brain (Pax-2, -5), the derivatives of the third and fourth pharyngeal pouches (Pax-9), and the neural crest derivatives (Pax-3, -7). Moreover, a remarkable characteristic of mutations at Pax gene loci is that the loss of one allele is sufficient to exhibit a dominant phenotype, also known as
haploinsufficiency. Although haploinsufficiency itself is a rare case among vertebrate transcription factors (Engelkamp and van Heyningen, 1996), Pax proteins seem to exhibit a remarkable degree of dosage sensitivity. Therefore, a correct dosage of these Pax proteins appears to be essential to ensure normal development.

### 3.6.1. Naturally occurring Pax mutants

The importance of Pax genes for embryonic development is emphasized by the fact that naturally occurring mutations in Pax genes have been associated with congenital abnormalities in mouse and man (Dahl et al., 1997; Hill and Hanson, 1992; Read, 1995; summarized in Table 1). In most cases, an obvious correlation between the expression patterns of Pax genes and the manifestation of abnormalities in the corresponding mutant phenotypes can be seen.

**Table 1. Diseases in mouse and man associated with Pax gene mutations**

<table>
<thead>
<tr>
<th>Pax genes</th>
<th>Mouse mutant (natural)</th>
<th>Human syndrome</th>
</tr>
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<tbody>
<tr>
<td>Pax-1</td>
<td>undulated (un/un\textsuperscript{ex}/Un\textsuperscript{5})</td>
<td>Spina bifida (?)</td>
</tr>
<tr>
<td>Pax-3</td>
<td>Splotch</td>
<td>Waardenburg syndrome I and III</td>
</tr>
<tr>
<td>Pax-2</td>
<td>Pax-2\textsuperscript{1Neu}</td>
<td>Renal coloboma syndrome</td>
</tr>
<tr>
<td>Pax-8</td>
<td></td>
<td>Congenital hypothyroidism (CH)</td>
</tr>
<tr>
<td>Pax-6</td>
<td>Small eye (Sey)</td>
<td>Aniridia; Peter's anomaly</td>
</tr>
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(adapted from Dahl, 1997)

In mouse, the Pax-1 gene has been found to be mutated in three *undulated* alleles causing malformations of the vertebral column (Dahl et al., 1997; Wilm et al., 1998). The phenotypes range from mild skeletal abnormalities to severe skeletal malformations in the different heterozygous conditions. In *undulated* (un), an amino acid substitution in the paired domain alters the DNA binding affinity and specificity of the mutated protein (Ballinger et al., 1988; Chalepakis et al., 1991). The *undulated extensive* (un\textsuperscript{ex}) allele harbours a deletion that includes the last exon of the Pax-1 gene (Dietrich and Gruss, 1995). In the Undulated short-tail (Un\textsuperscript{s}) allele, the entire Pax-1 locus is absent (Wallin et al., 1996). In humans, it is not yet known whether mutations in
the Pax-1 gene are also associated with congenital diseases. Recent reports indicate, however, that mutations in the Pax-1 gene could contribute to the pathogenesis of *spina bifida* in mouse and human (Helwig et al., 1995; Hol et al., 1996).

A deletion in the mouse Pax-3 gene causes the *Splotch* phenotype (Epstein et al., 1991). Homozygous *Splotch* mice show severe defects in closure of the neural tube (exencephaly and spina bifida), impaired limb muscle development and malformations in neural crest derivatives. Mutations in the human Pax-3 gene were found in patients with Waardenburg syndrome (Baldwin et al., 1992; Tassabehji et al., 1992; Tassabehji et al., 1993; Morell et al., 1992). This clinically and genetically heterogenous disorder is characterized by numerous defects in neural crest-derived tissues.

Eye defects found in species ranging from *Drosophila* to humans as a consequence of mutations in the Pax-6 gene illustrate the evolutionary conservation of Pax gene function in embryogenesis (Halder et al., 1995; Hanson and van Heyningen, 1995). In *Drosophila*, mutations in the gene homologous to Pax-6 are responsible for the *eyeless* phenotype (Quiring et al., 1994). In the mouse, homozygous *Small eye (Sey)* mutants completely lack eyes and nasal cavities, whereas heterozygous animals show a reduction in eye size (Hill et al., 1991). In the human syndrome aniridia, Pax-6 mutations range from single base pair mutations to large deletions (Hill et al., 1991; Ton et al., 1991). The patients suffer from complete or partial absence of the iris and defects in cornea, lens, retina, and optic nerve. Additionally, mutations at the Pax-6 locus in man have been described for Peter's anomaly (Hanson et al., 1994).

Recently, mutations have been also identified in genes of the Pax-2/5/8 class. Renal-coloboma syndrome has been correlated with heterozygosity for natural Pax-2 gene mutations in human families (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). In mouse, a spontaneous mutation of the Pax-2 gene (Pax-2<sup>Neu1</sup>) has been reported that cause a similar phenotype as described for patients with renal-coloboma syndrome (Favor et al., 1996). In human newborns, congenital hypothyroidism due to thyroid dysgenesis is a common disease. Mutations in the Pax-8 gene have been implicated in the pathogenesis of thyroid dysgenesis. Recently, patients with heterozygous Pax-8 mutations were described, which suffer from impaired thyroid gland function due to thyroid hypoplasia. Three separate Pax-8 mutations were identified which cause severe reduction in DNA binding and transactivation activity (Macchia et al., 1998).
3.6.2. Genetically engineered Pax mutants

Over the last few years, several new mouse strains have been generated by gene targeting methods to create defined Pax null alleles (Table 2).

Table 2. Phenotypes of homozygous mice with targeted deletions of Pax genes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Phenotype</th>
<th>References</th>
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<tr>
<td>Pax-1</td>
<td>Viable and fertile; skeletal abnormalities along the vertebral column, in the sternum and the scapula</td>
<td>Wilm et al. (1998)</td>
</tr>
<tr>
<td>Pax-9</td>
<td>Perinatal lethal; lack of derivatives of the third and fourth pharyngeal pouches (thymus, parathyroid gland, ultimobranchial body); absence of teeth; abnormalities of head and visceral skeleton; supernumerary digits; lack of the flexor of the hindlimb toes</td>
<td>Peters et al. (1998)</td>
</tr>
<tr>
<td>Pax-7</td>
<td>Postnatal lethal; dysgenesis of cephalic neural crest derivatives (malformations in facial structures involving the maxilla and nose)</td>
<td>Mansouri et al. (1996)</td>
</tr>
<tr>
<td>Pax-2</td>
<td>Embryonic lethal; lack of kidneys, ureters and genital tracts; eye defects (agenesis of the optic chiasma, optic nerve coloboma); inner ear defects (agenesis of the cochlea and the spiral ganglion); mouse strain dependent exencephaly (30-100%)</td>
<td>Torres et al. (1995); Torres et al. (1996); Schwarz et al. (1997)</td>
</tr>
<tr>
<td>Pax-5</td>
<td>Postnatal lethal; 5% viable and fertile; defect in B-cell development; mild defect of the posterior midbrain (reduction of the inferior colliculus) and altered foliation of the anterior cerebellum</td>
<td>Urbánek et al. (1994)</td>
</tr>
<tr>
<td>Pax-8</td>
<td>Postnatal lethal; thyroid gland defects (lack of thyroxine-producing follicular cells)</td>
<td>Mansouri et al. (1998)</td>
</tr>
<tr>
<td>Pax-4</td>
<td>Postnatal lethal; pancreas defects (lack of mature insulin- and somatostatin-producing cells)</td>
<td>Sosa-Pineda et al. (1997)</td>
</tr>
<tr>
<td>Pax-6</td>
<td>Perinatal lethal; lack of eyes and olfactory bulbs; pancreas defects (lack of mature glucagon-producing cells)</td>
<td>St-Onge et al. (1997)</td>
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3.7. Pax genes in the development of the midbrain-hindbrain boundary

The midbrain-hindbrain boundary (MHB, mesencephalic-metencephalic boundary, or isthmus) represents an important signaling center that controls the establishment of specific brain structures during embryonic development (Joyner, 1996). The MHB has been identified as a major site of Pax gene expression during neural development. Expression of Pax-2, Pax-5, and Pax-8 overlaps spatially and temporally at the MHB with Pax-2 being the first gene and Pax-8 being the latest to be expressed (Asano and Gruss, 1992; Krauss et al., 1991; Nornes et al., 1990; Pfeffer et al., 1998).

3.7.1. Development of the vertebrate central nervous system

In vertebrate embryos, the central nervous system forms along the dorsal midline of the body axis. Its formation is the consequence of a series of inductive interactions and is initiated during gastrulation, when dorsal mesodermal cells moves into contact with the overlying ectoderm. In this process, known as neural induction, mesodermal cells influence ectodermal cells to adopt a neural fate. Subsequently, the neural ectoderm folds up to form the neural tube and regionalizes along its anteroposterior axis in response to inductive signals emanating from the dorsal mesoderm or produced in restricted region of the developing neural epithelium (Kelly and Melton, 1995; Lumsden and Krumlauf, 1996). The appearance of three swellings (vesicles), named prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain), in the rostral neural tube is the first morphological indication of a segmented organization of the developing brain. The prosencephalon is then divided into the telencephalon and the diencephalon, and later into smaller prosomers (Rubenstein et al., 1994). The rhombencephalon is further divided into the metencephalon and myelencephalon, with the latter being further segmented into smaller rhombomeres. In contrast, the mesencephalon and metencephalon do not appear to be further segmented (Joyner, 1996).

Chick-quail homotypic and heterotypic transplantation studies have given insights in cell fate and cell commitment of distinct brain regions (Wassef and Joyner, 1997). They have revealed that the cerebellum is derived from both the caudal mesencephalon and the metencephalon, whereas the tegmentum and colliculi (tectum) are derived from more rostral regions of the mesencephalon. Interestingly, the MHB has been identified as an organizer center controlling the development of the surrounding neuroepithelium. Tissue grafts from the MHB
region were shown to induce an ectopic midbrain or cerebellum when transplanted into the chicken forebrain or hindbrain, respectively (Bally-Cuif and Wassef, 1995).

3.7.2. Signaling events in the development of the MHB

A large effort has been directed towards identification of regulatory genes to gain insight into the underlying molecular events that comprise the complex set of intrinsic and extrinsic instructions that pattern the brain. Over the past decade, a number of molecules with supposed regulatory and signaling functions required for the proper development of the MHB territory has been identified (reviewed in Joyner, 1996; Wassef and Joyner, 1997). Members of the Engrailed (En), Pax, Wnt and FGF gene families are expressed in spatially restricted domains during early development of the MHB, most of them being homologues of Drosophila segmentation genes. En-1, En-2, Wnt-1, FGF-8, Pax-2, Pax-5 and Pax-8 are transiently expressed in overlapping domains surrounding the midbrain-hindbrain junction (Chalepakis et al., 1993; Davis and Joyner, 1988; Rowitch and McMahon, 1995). The present understanding of their function in MHB development is mainly based on the analysis of genetically engineered mouse mutants. Targeted mutations of these genes revealed that loss-of-function causes similar abnormalities of variable strength in early brain development. These genes appear to be involved in early specifications, proliferation, and/or survival of the ventricular layer cells that give rise to the MHB derivatives. Variations in phenotype severity might be a consequence of differences in spatial and temporal gene expression and redundant gene function among gene family members. Furthermore, gene disruption studies in zebrafish and gain-of-function experiments in chicken have contributed to elucidate molecular mechanisms underlying MHB development.

3.7.2.1. Consequences of Pax-2/5/8 gene disruptions for MHB development in the mouse

Inactivation of murine Pax-5 leads only to a mildly abnormal development of the anterior midbrain and the posterior cerebellum (Urbánek et al., 1994). Pax-2 homozygous mutant mice also develop essentially normal MHB derivatives (Schwarz et al., 1997). However, analysis of double Pax-2/Pax-5 mutants reveals a more severe phenotype than that obtained in single mutants suggesting functional redundancy between the two gene family members (Schwarz et al., 1997). Analysis of Pax-8 homozygous mutants does not reveal
any defects in MHB derivatives implicating compensatory function of Pax-2 and/or Pax-5 in the mutant animals (Mansouri et al., 1998).

3.7.2.2. Consequences of Pax-2.1 gene disruption for MHB development in the zebrafish

In contrast to the homozygous Pax-2 mouse mutants, chemically induced inactivation of the zebrafish Pax-2.1 gene shows a severe phenotype in zebrafish embryos. The noi (no isthmus) mutant is characterized by the loss of the midbrain and cerebellum (Brand et al., 1996; Lun and Brand, 1998). A similar phenotype with malformations at the MHB can be induced by injection of antibodies raised against the Pax-2.1 protein (Krauss et al., 1992). During zebrafish embryogenesis, Pax-2.1 expression at the MHB precedes that of Pax-5 and Pax-8. Noteworthy, Pax-5 and Pax-8 expression at the MHB strictly depends on Pax-2.1 function. Therefore, inactivation of Pax-2.1 can be considered as a functionally equivalent to triple inactivation of Pax-2/5/8 genes. These findings explain the phenotypic differences between mice and zebrafish Pax-2.1 mutants (Brand et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998).

3.7.2.3. Consequences of altering gene dosage of other factors for MHB development

Both, Wnt-1 and En-1 deficient mice have deletions of the cerebellum and the midbrain with Wnt-1 deficiency resulting in a more severe phenotype (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Wurst et al., 1994). En-2 mutants show a reduction in size of the cerebellum with an abnormal folding pattern and alterations of the colliculi (Millen et al., 1994). En-1/En-2 double mutants have a more severe deletion phenotype than the single mutants consistent with compensatory function between the two En proteins (Hanks et al., 1995). FGF-8 gene inactivation in mice causes cerebellar and midbrain defects (Meyers et al., 1998). Conversely, beads soaked in recombinant FGF-8 protein and implanted in the caudal forebrain can mimic the effect of MHB grafts by inducing ectopic midbrain structures in chick embryos (Crossley et al., 1996).
3.7.2.4. Conclusions

Analysis of mouse and zebrafish mutants has enabled the identification of many transcription factors and secreted molecules that function in the development of the MHB territory. Nevertheless, the hierarchies of interactions of these factors and the molecular mechanisms which allow the establishment, the further subdivision, and full development of the complement of midbrain and cerebellar structures are still poorly understood. One reason for the difficulty to dissect the complexity of the developmental program in mutant animals is the functional redundancy observed for several components. Therefore, approaches with multiple experimental strategies and model systems will shed more light on the unresolved questions.

3.8. Pax genes in kidney organogenesis

3.8.1. Development of the vertebrate excretory system

During vertebrate embryogenesis, the excretory system develops from the intermediate mesoderm by successive formation of three distinct kidneys along the rostrocaudal axis of the embryo, namely the pronephric, the mesonephric, and the metanephric kidney (Saxén, 1987) (Fig. 2).

Vertebrate kidney organogenesis is initiated by induction of the pronephric kidney. The pronephric kidney consisting of the pronephros and the pronephric duct (also known as nephric or Wolffian duct) is established as the earliest kidney and represents the simplest form of an excretory organ in vertebrates. In embryos of fish and amphibians, the pronephric kidney develops to a fully functional organ, while it remains a transient structure in higher vertebrates. The pronephric duct constitutes the central component of the excretory system throughout development (Herzlinger, 1995). It is the drainage channel of the functional pronephric and mesonephric kidney, it gives rise to the ureteric bud of the metanephros and ultimately contributes to the male genital system as the ductus deferens. Moreover, the epithelium of the pronephric duct and its derivatives induce the morphogenesis and differentiation of the mesonephric and metanephric mesenchymal nephric cord into tubular structures. In fish and amphibians, the mesonephric kidney represents the excretory organ of the adult animal. In reptiles, birds and mammals, however, the mesonephric kidney is succeed by the metanephric kidney. Development of the metanephric kidney is initiated when the ureteric bud induces nephrogenic mesenchymal cells to condense and create an epithelial aggregate that will give rise to the renal nephron. As an example of reciprocal induction, the condensed mesenchylene
will induce the ureteric bud to branch repeatedly to form the renal collecting ducts and the ureter (Grobstein, 1955).

Fig. 2. Overall scheme of vertebrate kidney development. (A) The pronephros and the nephric duct form from the nephrogenic cord. (B) The pronephros degenerates, whereas the elongating nephric duct induces by interacting with the nephrogenic cord the formation of the mesonephros and remains as the drainage channel of the functional mesonephros. (C) Development of the metanephric kidney is initiated by the ureteric bud, an outgrowth of the caudal aspect of the nephric duct that induces the nephrogenic mesenchyme to condense. The mesonephros also degenerates but is still seen during early stages of the metanephros development. Figure taken from Burns (1955).

3.8.2. The pronephric kidney as a model system to study kidney organogenesis

Based on histology, morphology and classical experimental embryology (Fox, 1963; Vize et al., 1995; Vize et al., 1997), it appears that the formation of the pronephric kidney requires many of the same mechanisms necessary for
Introduction

later steps of kidney organogenesis such as inductive interactions, mesenchyme-to-epithelial transitions, cell migration and patterning formation along a tubular system. All three kidneys have a similar functional organization and differ mostly in the spatial assembly within the organism and the number and types of nephrons (Saxén, 1987; Vize et al., 1995; Vize et al., 1997). While the metanephric kidney of humans contains up to 1 million nephrons, the pronephric kidney of amphibians is typically composed of 2-3 nephrons. The nephron is the structural and functional unit of the vertebrate excretory system ensuring excretion and osmoregulation. Each nephron consists of a glomerulus and a segmented tubular system. In the pronephros, however, a single vascularized glomus replaces individual glomeruli. The glomus filters waste from the blood into the nephrocoel or coelom. Associated coiled pronephric tubules collect the filtrate through ciliated funnels (nephrostomes) and return resorbed materials to the blood. The filtrate remaining after completion of tubular resorption is disposed as urine via the pronephric duct (Fig. 3). Bilateral removal of the pronephroi in embryos of lower vertebrates results in edema and death, whereas unilateral extirpation leads to compensatory hypertrophy of the remaining pronephros (Fales, 1935; Holtfreter, 1944; Howland, 1927).

For the analysis of metanephric kidney development, the use of an in vitro organ culture system and genetic evidence provided by transgenic animal models have helped to define essential components of an emerging network of signaling pathways that regulates its development (Ekblom, 1996; Herzlinger, 1995; Lechner and Dressler, 1997; Vainio and Müller, 1997). Interestingly, genes that have been identified to be critical in normal metanephric kidney development are also expressed in the pronephros or in the early mesoderm (Davies and Brändli, 1996; Vize et al., 1997) indicating that these genes play similar roles in the development of all three kidneys. Taken together, the pronephric kidney can serve as an simplified organ model to study the molecular and cellular basis of vertebrate kidney formation.

3.8.3. Expression of Pax genes in the developing excretory system

Expression of Pax-2 and Pax-8 is associated with all three stages of kidney organogenesis. In the pronephric kidney, the presence of Pax-2 and Pax-8 has been reported in human, mouse and zebrafish (Dressler and Douglass, 1992; Krauss et al., 1991; Pfeffer et al., 1998; Terzic et al., 1998). During mesonephric development, Pax-2 transcripts are observed in the mesonephric tubules and in the Wolffian duct. Once the mesonephros
degenerates, Pax-2 expression is maintained in the ureteric bud, an outgrowth of the caudal aspect of the Wolffian duct, and is activated in the condensing metanephric mesenchyme and its early epithelial derivatives. Expression is rapidly downregulated as terminal differentiation of the renal tubules proceeds (Dressler and Douglass, 1992; Eccles et al., 1992). In contrast, Pax-8 expression is restricted to more differentiated stages in mesonephric and metanephric development. During metanephric kidney organogenesis, Pax-8 expression first appears in renal vesicles, comma- and S-shaped bodies, but is absent in the ureteric bud and condensing mesenchyme (Poleev et al., 1992).

**Fig. 3.** Organization of the pronephric kidney. Wastes filtered from the glomus into the coelom are collected by the tubules via the nephrostomes. Resorbed materials are returned to the blood stream via the pronephric sinus (blue) which is derived from the posterior cardinal vein and surrounds the tubules. Filtrate remaining after tubular resorption is disposed as urine via the pronephric duct. Figure taken from Vize et al. (1997).
3.8.4. Functions of Pax genes in kidney development

Strict control of Pax-2 expression levels appears to be important for normal metanephric development. During metanephric kidney organogenesis, Pax-2 is rapidly downregulated as terminal differentiation of the renal epithelium proceeds (Dressler et al., 1990; Dressler and Douglass, 1992; Eccles et al., 1992). Failure to repress Pax-2 in transgenic mice results in severe kidney abnormalities presumably by interfering with terminal differentiation of renal epithelial cells (Dressler et al., 1993). Downregulation of Pax-2 is believed to be partially mediated by Wilms' tumor suppressor (WT-1). A significant increase in WT-1 protein levels coincides precisely with downregulation of Pax-2 expression in precursor cells of the glomerular epithelium. Indeed, WT-1 directly inhibits Pax-2 transcription in vitro (Ryan et al., 1995). Furthermore, both Pax-2 and Pax-8 are expressed at elevated level in undifferentiated mesenchymal cells of Wilms' tumor (Dressler and Douglass, 1992; Eccles et al., 1995), a pediatric tumor of the kidney that is associated with mutations in WT-1 (reviewed by Coppes, 1995). Vice versa, the WT-1 promoter is actively stimulated when cotransfected with Pax-2 in vitro. Deletion mutagenesis of the WT-1 promoter identified potential binding sites for Pax-2 (Dehbi et al., 1996; McConnell et al., 1997). Overexpression of Pax-2 in human kidney cells (HEK293) leads to a 2-fold increase of WT-1 expression (Torban and Goodyer, 1998). Together, these findings suggest a regulatory mechanism by which WT-1 and Pax-2 control each other's expression.

In vitro experiments using Pax-2 antisense oligonucleotides in kidney organ cultures have indicated that Pax-2 is required for mesenchyme-to-epithelium conversion in metanephric tubule formation (Rothenpieler and Dressler, 1993). With significantly reduced Pax-2 protein levels, mesenchymal kidney cells fail to aggregate and do not undergo sequential morphological changes characteristic for epithelial cell formation. Overexpression studies of Pax-2 in human embryonic kidney cells (HEK293) suggest that Pax-2 promotes the mesenchyme-to-epithelium transition by modifying the expression of genes critical to the epithelial phenotype. Elevated levels of Pax-2 protein lead to upregulation of E-cadherin and suppression of vimentin expression (Torban and Goodyer, 1998). Taken together, the in vitro studies have enabled the identification of possible molecular interactions and the elucidation of some of the functional aspects of Pax-2 during metanephric development.
Introduction

3.8.4.1. Consequences of Pax-2 mutations

Additional insights into the role of Pax genes in kidney development have emerged from genetic analysis in zebrafish, mice and humans. To date, several human and three mouse Pax-2 mutations have been described. Heterozygous carriers of these mutations express eye and kidney abnormalities with a variability in expressivity. In addition, zebrafish with mutations in the Pax-2.1 gene have been recently reported.

*Human Pax-2 mutations cause renal-coloboma syndrome*

The renal-coloboma syndrome (optic nerve coloboma-renal disease, papillo-renal syndrome) is a rare disorder characterized by autosomal dominant inheritance of optic, auditory, and renal defects (Weaver et al., 1988). Ocular manifestations in this syndrome are variable anomalies of retinal and optic disk dysplasia. Anomalies of the optic nerve and thinning of the retinal epithelium results in loss of visual acuity and defective visual fields. Patients suffer simultaneously from chronic renal failure, chronic glomerulonephritis and/or renal hypoplasia. Occasionally, the renal-coloboma syndrome is associated with hearing defects.

Recently, renal-coloboma syndrome has been correlated with heterozygosity for natural Pax-2 gene mutations in unrelated human families (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). Over the last three years, analysis of patients with renal-coloboma syndrome has led to the identification of at least six mutations (for review, see Online Mendelian Inheritance in Man (OMIM) homepage; http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/). Both, truncating mutations as well as missense mutations in the human Pax-2 gene have been identified (summarized in Fig. 4).

Two independent insertions of a single base pair within the paired domain (exon 2) lead to frameshift mutations resulting in severely truncated proteins (Sanyanusin et al., 1995a; Schimmenti et al., 1997). Another frame shift mutation in the paired domain is caused by a deletion of 22 bp in exon 2 (Schimmenti et al., 1997). Furthermore, individuals of other affected families carry a deletion of a single nucleotide in the octapeptide (exon 5) that results in a truncated protein which lacks the partial homeodomain and the entire C-terminus (Sanyanusin et al., 1995b; Schimmenti et al., 1995). More recently, a heterozygous missense mutation and a heterozygous hexanucleotide duplication in the paired domain (exon 3) of the Pax-2 gene of unrelated
families have been described that do not lead to truncated proteins (Devriendt et al., 1998).

![Diagram of Pax-2 protein mutations](image)

**Fig. 4.** Depiction of the location of the mutations within the human Pax-2 protein causing renal-coloboma syndrome. The structure of the Pax-2 protein is shown schematically with the corresponding domain organization (PD, paired domain; OP, octapeptide motif; HD, homeodomain). Exons numbered from 1 to 10 according to Pfeffer et al. (1998) are present in all Pax-2/5/8 members, whereas the suffix '1.' refers to less well conserved, non-canonical exons. Above, positions of the missense mutations are indicated that preserve the original reading frame of the Pax-2 protein. Below, positions of mutations are indicated that introduce frame shifts resulting in truncated Pax-2 proteins.

In principle, these mutant Pax-2 proteins can lead to a disruption of transcription factor complexes, or to a competitive inhibition of DNA binding of the normal Pax-2 protein. The frame shift mutations in the paired domain of Pax-2 causing severely truncated proteins with a lack of DNA binding and transactivation activities can be regarded as loss-of-function mutations. The missense mutations in the paired domain are expected to result in abnormal folding of the Pax-2 protein that might result in reduction or loss of DNA binding and transactivation activity. The frame shift mutation in the octapeptide motif leads to a truncated protein with an intact paired domain that would still be able to bind DNA but obviously lacks the ability to transactivate the expression of target genes. In summary, the reduced dosage of functional Pax-2 protein appears to be at the basis of the eye and renal malformations.
Pax-2 mutations and vesicoureteric reflux

Vesicoureteric reflux (VUR) is a common childhood condition characterized by regurgitation of urine from the bladder to the kidney (Bailey, 1979) causing severe kidney damage (reflux nephropathy) and even leading to end stage renal failure in a significantly high percentage of patients (Bailey et al., 1984). VUR is a result of a congenital defect of the length, diameter, musculature or innervation of the submucosal segment of the ureter (Stephens and Lenaghan, 1962). A developmental abnormality of the caudal portion of the ureteric bud is believed to be the primary defect (Stephens, 1976). VUR is frequently clustered in families (Heale et al., 1979) suggesting its genetic origin. Normally, hereditary VUR is not associated with other anomalies (primary familial VUR), but in rare instances VUR occurs in association with other diseases, such as the renal-coloboma syndrome, which is caused by a Pax-2 gene mutation (Sanyanusin et al., 1995b). An extensive survey of patients suffering from VUR by single stranded conformational polymorphism analysis revealed no alteration in the Pax-2 gene. In summary, Pax-2 mutations are not considered as a major cause of primary familial VUR (Choi et al., 1998; Eccles et al., 1996).

Murine Pax-2 mutants

Three mouse strains with mutations in the Pax-2 locus have been described. The Krd (kidney and retinal defects) strain carries a large transgene induced chromosomal deletion (~7 cM) including the Pax-2 locus. They develop retinal abnormalities and a high incidence of kidney defects (hypoplasia) (Keller et al., 1994). Although these phenotypic features observed in the Krd mouse might be attributed also to the loss of other genes, the effect is dominant and resembles that observed in human patients heterozygous for a Pax-2 gene mutation.

Torres et al. (1995) generated a Pax-2 knock out mutation in the mouse. Heterozygous mutants frequently showed reduction in kidney size. The analysis of homozygous mouse mutants indicates that Pax-2 controls multiple steps during urogenital development as demonstrated by the absence of mesonephric and metanephric kidneys, ureters and genital tracts (Torres et al., 1995). The phenotype has led to the proposal that Pax-2 might be a primary regulator for these developmental processes. Similar to humans, Pax-2 mutant mice show eye (optic nerve coloboma) and inner ear defects (Torres et al., 1996).
Favor et al. (1996) identified a spontaneous murine frameshift mutation (Pax2\textsuperscript{1Neu}) with a 1-bp insertion in the Pax-2 gene identical to that found in humans (Sanyanusin et al., 1995a). Heterozygous mutant embryos display a similar phenotype as described for human families with renal-coloboma syndrome by exhibiting defects in the kidney, the optic nerve, and the retinal layer of the eye. The homozygous animals reveal similar phenotypes as observed in the knock out mutants.

**Zebrafish Pax-2 mutants**

In zebrafish, two Pax-2 like genes called Pax-2.1 and Pax-2.2, respectively, have been identified (Krauss et al., 1991; Pfeffer et al., 1998). Similar to mouse Pax-2, zebrafish Pax-2.1 is expressed during kidney organogenesis as well as in the developing MHB, spinal cord, eye, and ear. Pax-2.2 differs from Pax-2.1 by the absence of expression in the nephric system and by a delayed onset of transcription in other tissues with Pax-2.1 expression. The chemically induced no isthmus (noi) mutations is known to result from mutations in the Pax-2.1 gene (Brand et al., 1996; Lun and Brand, 1998). During embryogenesis, mutant embryos form rudimentary ducts that fail to elongate and subsequently degenerate (Brand et al., 1996).

### 3.8.4.2. Consequences of Pax-8 mutations

Patients heterozygous for mutations in the Pax-8 gene display severe hypothyroidism, characterized by a dramatic reduction of the thyroid gland (Macchia et al., 1998). Inactivation of murine Pax-8 leads to thyroid dysgenesis due to the lack of follicular cells. However, no defects have been observed in other structures expressing Pax-8 (Mansouri et al., 1998). Although Pax-8 is expressed both during early and late vertebrate kidney development, mutations in the Pax-8 gene of mice and man affect obviously only the organogenesis of the thyroid gland and do not lead to kidney defects probably due to partially redundant function provided by Pax-2 during kidney organogenesis.

### 3.8.4.3. Conclusions

Overall, animals deficient in Pax genes are powerful tools to study Pax gene function in kidney organogenesis. Determination of the role of Pax genes in kidney development is however hampered by restricted experimental access in mouse and man. Furthermore, the interpretation of Pax gene function
analysis becomes complex due to functional redundancy and alternative splicing. Complementary animal models like *Xenopus laevis* and zebrafish may therefore provide novel insights concerning the role of Pax genes in kidney organogenesis.

### 3.9. *Xenopus laevis* as a novel model for the analysis of kidney organogenesis

Given its transient nature in higher vertebrates, pronephric kidney organogenesis is ideally studied in lower vertebrates. In fact, the pronephric kidney was first described in toad and frog embryos at the beginning of the nineteenth century (Müller, 1829). In many areas of developmental biology, the frog *Xenopus laevis* has become the experimental organism of choice over the last four decades. Despite the lack of classical genetics, the major advantage of the *Xenopus* system is the possibility of generating thousands of embryos and the ease with which they can be manipulated by microdissection. In addition, simple microinjection techniques and novel transgenic methods (Kroll and Amaya, 1996) provide powerful tools to elucidate gene function.

In *Xenopus* embryos, the development of the pronephric kidney into a functional organ occurs very rapidly and is completed within 2-3 days after fertilization (Nieuwkoop and Faber, 1994). The availability of a suitable *in vitro* organ culture system was essential in order to define the inductive interaction occurring during metanephric development (Saxén, 1987). Interestingly, the induction and formation of pronephric tubules can be reproduced *in vitro* with animal cap cultures (explants of blastula ectoderm) treated with activin and retinoic acid (Uochi and Asashima, 1996). Over the last three years, our laboratory has identified over 30 genes with expression in the pronephric kidney of *Xenopus laevis* (Davies and Brändli, 1996; Brändli laboratory, unpublished data). This large collection of marker genes enables an extensive analysis of the molecular cascades acting during pronephric kidney development. Overall, the *Xenopus* embryo represents therefore a promising model for *in vivo* as well as *in vitro* studies to unravel the molecular and cellular basis of early kidney organogenesis.
4. Aim of the thesis

Pax genes have been implicated as key regulators of organ development in the vertebrate embryo. The aim of the present thesis is to gain further insight into the function of Pax-2/5/8 gene family members in early kidney organogenesis using the amphibian *Xenopus laevis* as a model. The objectives of the thesis are:

- to isolate and sequence cDNAs encoding all three *Xenopus* Pax-2/5/8 orthologues;

- to characterize the temporal and spatial expression of Pax-2/5/8 transcripts during *Xenopus* embryogenesis;

- to characterize the spectrum of alternative spliced Pax-2 transcripts in pronephric kidneys, animal cap cultures, and in whole embryos; and

- to assess the functional properties of different Pax-2 isoforms in pronephric kidney organogenesis and MHB development.

It is anticipated that the proposed studies will expand our understanding of how Pax genes control tissue formation and organogenesis. More specifically, a better knowledge of the molecular mechanisms underlying kidney organogenesis are of central importance in understanding the molecular basis of inherited renal diseases, such as the renal-coloboma syndrome. This will subsequently allow the development of novel diagnostic methods and serve as a starting point for improved therapeutic strategies.
5. Results

5.1. *Xenopus* Pax-2 displays multiple splice forms during embryogenesis and pronephric kidney development

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5.1.1. Abstract

Kidney organogenesis is initiated with the formation of the pronephric kidney and requires Pax-2 gene function. We report here the cloning and characterization of Pax-2 cDNAs from the frog *Xenopus laevis*, a model system suitable to study early kidney organogenesis. We show that expression of *Xenopus* Pax-2 (XPax-2) genes was confined to the nervous system, sensory organs, the visceral arches, and the developing excretory system. DNA sequencing of XPax-2 cDNAs isolated from head and pronephric kidney libraries revealed seven novel alternatively spliced Pax-2 isoforms. They all retain DNA-binding domains, but can differ significantly in their C-termini with some isoforms containing a novel Pax-2 exon. We investigated the spectrum of XPax-2 splice events in pronephric kidneys, animal cap cultures and in whole embryos. Splicing of XPax-2 transcripts was found to be extensive and temporally regulated during *Xenopus* embryogenesis. Since all investigated tissues expressed essentially the full spectrum of XPax-2 splice variants, we conclude that splicing of XPax-2 transcripts does not occur in a tissue-specific manner.
5.1.2. Introduction

The development of the vertebrate excretory system is characterized by the successive formation of three distinct kidneys, the pronephros, the mesonephros and the metanephros (Saxén, 1987). All three kidneys are derived from the intermediate mesoderm and appear in a precise temporal and spatial sequence during embryogenesis. The pronephros is established as the earliest and most anterior kidney and represents the simplest form of an excretory organ in vertebrates. Its structural organization consisting of two components, a tubular system and an excretory duct, has already been recognized in the early nineteenth century through anatomical studies of amphibian embryos (Müller, 1829). As with all kidneys, the basic excretory unit of the pronephric kidney is the nephron. Each nephron is initially composed of a glomerulus and a segmented tubular system. As development proceeds, the pronephric glomeruli fuse to a single vascularized glomus. Pronephric tubules are lined by diverse epithelial cell types and are connected at their proximal ends to the coelomic cavity via ciliated funnels (nephrostomes). Individual nephrons are linked together through connecting tubules, which fuse to a single broad collecting tubule that communicates with the pronephric (or Wolffian) duct (Fox, 1963; Fraser, 1950). The organization of pronephric nephrons is therefore very much like that of the more advanced mesonephric and metanephric kidneys. A major difference lies in the number of nephrons that comprises the different kidney types. While the metanephric kidney of humans can contain up to 1 million nephrons, the pronephric kidney of amphibians is typically composed of 2-3 nephrons. This simple structural organization makes the pronephric kidney an attractive system to study kidney organogenesis (Fox, 1963; Vize et al., 1995).

Maturation of the pronephric kidney into a functional excretory organ is restricted to larval stages of fish and amphibians. It mediates the excretion of metabolic end products from the blood, the reabsorption of ions, and the control of water balance, and is therefore essential for the survival of the embryo (Fox, 1963). In higher vertebrates, pronephric tubule differentiation remains rudimentary, but pronephric duct formation proceeds normally (Burns, 1955; Saxén, 1987). The pronephric duct and its derivatives participate in the induction of both the mesonephric and metanephric kidneys of vertebrate embryos (Burns, 1955; Herzlinger, 1995; Saxén, 1987). In amphibian embryos, the isolated pronephric duct has been shown to act in vitro as a strong inducer of mesonephric tubules if combined with explants of mesonephric mesenchyme (Etheridge, 1968). Similarly, induction of the metanephric kidney is dependent
Results

on the ureteric bud, an epithelial diverticulum that forms from the caudal aspects of the pronephric duct. Ablation of the ureteric bud impairs metanephric kidney formation (Grobstein, 1955; Gruenwald, 1952; Saxén, 1987). While the role of the pronephric duct as an inducer of the mesonephric and metanephric kidneys is clearly established, it is currently not known whether a similar function is also necessary for proper pronephric kidney development. Since pronephric duct and tubules arise from separate but adjacent populations of primordial cells of the intermediate mesoderm (O'Connor, 1938; Vize et al., 1995), it is however conceivable that inductive interactions exerted by the pronephric duct could also promote pronephric tubule differentiation.

The molecular mechanisms underlying kidney organogenesis are in general still poorly understood. An ever growing number of molecules with supposed regulatory and signaling functions have been found to be expressed at various stages of kidney development (Davies and Brändli, 1996). The use of in vitro organ culture systems and genetic evidence provided by transgenic animal models has helped to define essential components of an emerging network of signaling pathways that regulate metanephric kidney development (Ekblom, 1996; Herzlinger, 1995; Lechner and Dressler, 1997). These components include growth factors of the Wingless/int (e.g. Wnt-4), transforming growth factor-ß (e.g. GDNF), and bone morphogenetic protein (e.g. BMP-7) families; adhesion molecules (e.g. integrin α8), receptor tyrosine kinases (e.g. c-Ret), and transcriptions factors (e.g. BF-2, Emx-2 and WT-1). None of these gene products however appear to be absolutely necessary for normal pronephric and mesonephric kidney development. Thus, the nature of the molecules regulating early stages of kidney organogenesis remains still unresolved.

Transcription factors of the Pax class comprise a small, but important family of developmental control genes (Chalepakis et al., 1993; Mansouri et al., 1996; Stuart et al., 1994). Pax genes are defined by a 128-amino acid DNA binding domain, called the paired box domain, which is conserved in animal species ranging from nematodes to humans, and was originally identified in the Drosophila segmentation gene paired (Bopp et al., 1986). Based on sequence homology and the presence of class-specific amino acids in certain positions, the known paired domains can be divided into six different classes (Noll, 1993; Nornes et al., 1996; Walther et al., 1991). Each member of the family shows spatially and temporally restricted expression patterns during embryonic development, with the majority of Pax genes being expressed in the central nervous system and/or the paraxial mesoderm and its derivatives (Chalepakis
Pax-2 is of particular interest with respect to kidney organogenesis. Pax-2 together with Pax-5 and Pax-8 comprise the class III Pax gene family (Walther et al., 1991). They contain an octapeptide motif and only a partial homeodomain. Pax-2 gene expression is associated with the earliest stages of kidney organogenesis (Dressler et al., 1990; Kadesh, 1992; Krauss et al., 1991; Mikkola et al., 1992; Püschel et al., 1992). Transcripts of Pax-2 are initially present in the pro- and mesonephric tubules, as well as in the pronephric duct and its derivatives. Later, Pax-2 expression is found in the condensing metanephric mesenchyme, its early epithelial derivatives, and in the collecting duct epithelium. Pax-2 transcripts are however never associated with the undifferentiated mesenchyme.

A number of mutations in the Pax-2 gene reported recently establishes a central role for Pax-2 in kidney organogenesis. In two human families, heterozygosity for Pax-2 mutations cause renal-coloboma syndrome (Sanyanusin et al., 1995a; Sanyanusin et al., 1995b). Patients display eye defects and kidney hypoplasia. The mutations appear to be loss-of-function mutations implying a dosage-sensitivity, haplo-insufficiency, for Pax-2. In mice, three strains with mutation in the Pax-2 gene have been reported. The Krd strain carries a large chromosomal deletion affecting several genes including the Pax-2 locus (Keller et al., 1994). Torres et al. (1995) have generated Pax-2-deficient mice by targeted mutagenesis. A spontaneous mouse mutant carrying the Pax-2\(^{1}\)Neu mutation was found to be identical to a previously described human Pax-2 mutation (Favor et al., 1996). All three mice mutants show that a partial loss of Pax-2 gene dosage results in similar renal defects as in humans (Favor et al., 1996; Keller et al., 1994; Torres et al., 1995). Animals homozygous for mutations in the Pax-2 gene are more severely affected and completely lack mesonephric and metanephric kidneys, ureters, and genital tracts (Favor et al., 1996; Torres et al., 1995). The pronephric duct develops only rudimentary, and then degenerates at the point when it should begin to interact with the nephrogenic mesenchyme. Outside the excretory system, defects are also seen with neural tube closure and the development of the optic nerve, the inner ear, and structures at the midbrain-hindbrain boundary (Favor et al., 1996; Sanyanusin et al., 1995a; Sanyanusin et al., 1995b; Torres et al., 1995). Recently, defects in nervous system, sensory organs and kidneys were reported for zebrafish strains harboring mutations in the no-isthmus (noi) gene which encodes a possible homologue of mammalian Pax-2 (Brand et al., 1996; Macdonald et al., 1997).
The nature of the kidney defects observed in homozygous mutant animals define Pax-2 as an essential component of the intermediate mesoderm necessary for normal pronephric kidney development. Being a transcription factor, Pax-2 may be directly controlling regulatory hierarchies of genes involved in this process. To address the mechanism of Pax-2 function in an experimental system suitable for studies on early kidney organogenesis, we have isolated cDNAs encoding seven novel isoforms of Pax-2 from *Xenopus* head and pronephric kidney libraries. We document here the spatial pattern of *Xenopus* Pax-2 gene expression during embryogenesis with an emphasis on pronephric kidney development. Further, we assess the complexity of XPax-2 isoform expression in embryos, animal cap cultures and developing pronephric kidneys. We found that alternative splicing of XPax-2 transcripts was temporally regulated. The profiles of XPax-2 isoform expression were however similar for all tissues examined. Our results indicate that the mechanism controlling alternative splicing of XPax-2 transcripts during early *Xenopus* embryogenesis is not regulated in a tissue-specific manner.
5.1.3. Results

Cloning and sequence analysis of cDNAs encoding Pax-2 homologues from Xenopus laevis

The reverse transcription-polymerase chain reaction (RT-PCR) was chosen as a strategy to isolate a Xenopus Pax-2 (XPax-2) specific nucleic acid probe. Invariant sequence motifs within the highly conserved paired domain of class III Pax genes were selected to derive degenerate oligonucleotide primers (Fig. 5A). Sequence analysis of 88 bacterial clones containing subcloned RT-PCR generated cDNA fragments allowed us to distinguish five different classes. The majority of the analyzed sequences (>95%) displayed highest homology to human Pax-2 and could be divided into two closely related subclasses. The two subclasses represent transcripts of two pseudoallelic genes for Pax-2 in Xenopus laevis, which we have termed XPax-2a and XPax-2b. The remaining sequences displayed highest homology to either Pax-5, Pax-6, or Pax-9. Next, we used XPax-2-specific cDNA fragments to screen a Xenopus stage 28-30 head cDNA library (Hemmati-Brivanlou et al., 1991) in order to obtain cDNA sequences harboring a complete open reading frame. Three full-length cDNAs were isolated: XPax-2a(1), XPax-2a(3), and XPax-2b(2). DNA sequencing allowed us to assign two cDNAs as transcripts of the XPax-2a gene, while the third was derived from XPax-2b. An alignment of the predicted protein products is shown in Fig. 5A. The three XPax-2 proteins are essentially collinear with each other over the first 300 amino acids. Within this region, their amino acid sequences are almost identical (5 amino acids differences between the XPax-2a and XPax-2b proteins; 98.3% identity). This degree of identity is characteristic for gene products of pseudoallelic Xenopus genes (Graf and Kobel, 1991). Vertebrate class III Pax genes are defined by the presence of three conserved sequence motifs: the paired domain, the octapeptide, and a partial homeodomain (Walther et al., 1991). All three XPax-2 proteins were found to display the same characteristic sequence motifs (Fig. 5A). A comparison of these motifs with the three human class III Pax proteins consistently indicated highest homology to human Pax-2 (Fig. 6). For XPax-2a(1), the amino acid identity was found to be 100% within the paired domain and the octapeptide motif (not shown), and 96% for the partial homeodomain. The identity in the C-terminal domain to human Pax-2 was with 87% somewhat lower, but still significantly higher than to human Pax-5 and Pax-8.
Fig. 5. Amino acid sequences of Xenopus Pax-2 cDNAs and comparison of exon composition with human Pax-2 and Pax-8 splice variants. (A) Alignment of the deduced amino acid sequences of full-length Xenopus Pax-2 isoforms. The amino acid sequences are shown in single letter code. Gaps introduced to maximize similarity in the sequences are shown as dots. Amino acids differing between pseudoallelic Xenopus Pax-2a and Pax-2b isoforms are boxed. The amino acid sequence motifs recognized by the degenerate PCR primers XP-1 and XP-2, respectively, are indicated by arrows. The paired domain is shown with a black line, the octapeptide motif with a double line and the partial homeodomain with a broken black line. Asterisks denote the approximate locations of introns that were identified by sequencing of the human Pax-2 gene (Sanyanusin et al., 1996). (B) Comparison of Xenopus Pax-2 splice variants with human Pax-2 and Pax-8 isoforms. Only sequences down-stream of the octapeptide motif are shown. hPax-2(E6) represents a splice variant of human Pax-2 containing exon 6 (Sanyanusin et al., 1996). hPax-2(E10) includes exon 10, which results in an alteration of the reading frame and thereby produces an alternative C-terminus (Ward et al., 1994). The sequence of human Pax-8a was taken from (Polev et al., 1992). The exon organization of the coding sequence of human Pax-2 (Sanyanusin et al., 1996) is outlined beneath the sequence alignment. The exons of human Pax-2 are numbered from hE5 to hE12 and their boundaries are indicated by arrows. Note the gap between hE8 and hE9 generated by including XPax-2b(2) in the alignment. Human Pax-8a contains an alternatively spliced exon at the equivalent position.
Comparisons of Pax-2 proteins from zebrafish (previously referred to as Pax-B or \textit{pax[zf-b]}) and mouse to human Pax-2 revealed a similar degree of identity (Fig. 6). We conclude that XPax-2a and XPax-2b represent unambiguously \textit{Xenopus} homologues of the human Pax-2 gene.

**Paired domain**

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<tr>
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<td>99%</td>
<td>96%</td>
<td>92%</td>
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<tr>
<td>\textit{Xenopus} Pax-2a(1)</td>
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<td>97%</td>
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<td>zebrafish Pax-2</td>
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**Partial homeodomain**

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<td>67%</td>
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<tr>
<td>zebrafish Pax-2</td>
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**C-terminal domain**

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<td>zebrafish Pax-2</td>
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<td>74%</td>
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**Fig. 6.** Comparison of the deduced amino acid sequences of murine, \textit{Xenopus} and zebrafish Pax-2 with those of the human class III Pax proteins. The numbers indicate percent of identical amino acids within the domain examined. The paired domain covers a sequence motif of 128 amino acid residues (Bopp et al., 1986; Walther et al., 1991). The partial homeodomain is defined as 30-amino acid motif according to (Krauss et al., 1991). The C-terminal domain (137-140 amino acid residues in length) covers the entire region downstream of the partial homeodomain motif using human Pax-2 as a reference. To improve the alignment, intervening sequences generated by alternative splicing were removed from human Pax-8. The sequences were taken from the following sources: mouse Pax-2 (Dressler et al., 1990) with corrections according to (Adams et al., 1992); zebrafish Pax-2, originally described as \textit{pax[zf-b]} (Krauss et al., 1991); human Pax-2 (Eccles et al., 1992); human Pax-5 (Adams et al., 1992); human Pax-8 (Poleev et al., 1992).

Comparison of the C-terminal region of the XPax-2 cDNA sequences revealed that the extensive homologies in the open-reading frames are
interrupted by intervening nucleotide sequences downstream of the partial homeodomain (Fig. 5A). Relative to XPax-2a(1), XPax-2b(3) contains a 210 bp insertion, while XPax-2a(3) contains one of 99 bp. The extreme C-termini are again completely collinear. The intervening sequences do not alter the reading frame of the coding sequence. It is likely that these insertions represent alternative splice events during the maturation of XPax-2 transcripts. In humans, three Pax-2 variants generated by alternative splicing have been described (Eccles et al., 1992; Sanyanusin et al., 1996; Ward et al., 1994). Insertion of exon 6 between the octapeptide and the partial homeodomain generates a variant of human Pax-2, called here human Pax-2(E6) (Sanyanusin et al., 1996). Pax-2 isoforms homologous to human Pax-2(E6) have been described in zebrafish and mouse (Dressler et al., 1990; Krauss et al., 1991). Human Pax-2(E10) is generated by inclusion of exon 10. In this case, an alteration of the reading frame occurs and thereby produces a protein with an alternative C-terminus (Ward et al., 1994). We performed multiple sequence alignments to compare the variants of *Xenopus* Pax-2 with the known human Pax-2 isoforms (Fig. 5B). All three *Xenopus* Pax-2 isoforms lacked intervening sequences at a site equivalent to the exon 6 insertion in human Pax-2(E6). XPax-2a(1) was found to be homologous to the initially reported human Pax-2 protein (Eccles et al., 1992) which lacks exon 6 and exon 10 (data not shown). XPax-2a(3) is equivalent to human Pax-2(E10), with the exception that in *Xenopus* this alternative splicing event retains the original reading frame. XPax-2b(2) with its intervening sequence of 210 bp did not match any of the known vertebrate Pax-2 splice variants. The sequence alignment predicts that the alternative splice event occurs exactly at the junction of the human Pax-2 exon 8 and 9 (Fig. 5B). Transcripts of the related human Pax-8 gene are also subject to alternative splicing (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). Interestingly, a comparison of human Pax-8 and *Xenopus* Pax-2 isoforms revealed that XPax-2b(2) matched human Pax-8a including the novel intervening sequence (Fig. 5B). The alternatively spliced sequence of human Pax-8a is encoded by a separate exon (Kozmik et al., 1993; Poleev et al., 1995) and displays significant sequence homology to the *Xenopus* sequence (35.7% identity; 25 out of 70 amino acids). Taken together, the provided evidence suggests that the intervening sequence found in the C-terminus of XPax-2b(2) might be encoded by a novel Pax-2 exon.
Spatial expression of XPax-2 transcripts during early neural development, in sensory organs and visceral arches

We performed whole mount \textit{in situ} hybridization using digoxigenin-labeled RNA probes (Harland, 1991) to determine the spatial expression of XPax-2 in the developing Xenopus embryo. Several different probes were constructed and tested in hybridizations with consistent results. These include probes covering the entire coding sequence, as well as such that exclude the conserved paired domain of each XPax-2 isoform (data not shown; see Experimental Procedures for details). For maximal sensitivity, only results from hybridizations with probes directed against the entire coding sequence of XPax-2a(1) are shown (Fig. 7). Expression of localized XPax-2 transcripts was first detected at the end of gastrulation and was thereafter seen throughout embryogenesis (Fig. 7). Transcripts were essentially confined to the nervous system (midbrain, hindbrain, spinal cord), sensory organs (optic vesicle and stalk, otic vesicle), the visceral arches, and the excretory system (pronephros, pronephric duct, rectal diverticulum, proctodaeum).

In the developing Xenopus nervous system, the earliest site of localized XPax-2 transcription was detected at stage 13 (early neurula) in the anterior 1/3 of the neural plate (Fig. 7A,B). XPax-2 expression was associated with two distinct wedge-shaped patches of cells flanking the midline, separated by a small region devoid of expression. In the course of neural tube closure, the two patches of XPax-2 expression converge towards the dorsal midline (Fig. 7C, D). These cells will ultimately form the posterior portion of the midbrain at the midbrain-hindbrain boundary (Fig. 7K). Expression of XPax-2 in the hindbrain became discernible at stage 24 and was initially confined to a region posterior to the otic vesicle (Fig. 7F). Later, expression started to spread also anteriorly. Finally, XPax-2-expressing cells were found throughout the hindbrain (Fig. 7I). Transversal sections at the level of the hindbrain-spinal cord junction revealed XPax-2-expressing cells at a ventrolateral location in the neural tube (Fig. 7M). A first indication of Pax-2 expression associated with neurons of the developing spinal cord became apparent in embryos of stage 19 (Fig. 7D). Expression was initially confined to the anterior 1/3 of the spinal cord (Fig. 7E). As spinal cord differentiation proceeds in the posterior direction, additional clusters of cells expressing XPax-2 were detected. By stage 32, clusters of XPax-2 expressing cells were found extending along all but the most posterior part of the spinal cord (Fig. 7H). Dorsal inspection of stained embryos revealed that the Pax-2 expressing cells appeared as two longitudinal columns (Fig. 7D). By sectioning transversally through the spinal cord, we were able to show that cells...
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expressing XPax-2 were confined to an intermediate position along the dorsal-ventral axis of the spinal cord (data not shown). Based on this location, these cells most likely represent interneurons (Hartenstein, 1993; Roberts and Clarke, 1982).
Fig. 7. Spatial expression of XPax-2 transcripts during early *Xenopus* embryogenesis. Whole mount *in situ* hybridizations were performed on albino embryos using digoxigenin-labeled antisense RNA probes. Hybridization events were visualized as alkaline phosphatase chromogenic reaction products. Embryos stained in whole mount were used for sectioning and were cut at 70 μm (L, M) and 100 μM (N). (A, B) Lateral and dorsal views of a stage 13 embryo. XPax-2 expression is seen in two wedge-shaped patches of cells in the anterior 1/3 of the neural plate. Expression is absent at the midline (arrowhead). (C) Lateral view of a stage 19 embryo. XPax-2 expression can be detected in the regions of the future midbrain-hindbrain boundary and the optic stalk (arrowhead). (D) Dorsal view of a stage 19 embryo. Two rectangular-shaped patches of XPax-2 expressing tissues which will contribute to the midbrain-hindbrain boundary flank the midline (arrowhead). Note that staining of XPax-2 can now also be detected in single neurons arranged in stripes on either side of the midline. (E) Lateral view of a stage 21 embryo. XPax-2 expression is associated with the region of the future optic stalk (os) and vesicle, the midbrain-hindbrain boundary (m-h), the otic vesicle (ov) and with cells in the spinal cord (sc). Note also the first appearance of staining in the pronephric anlage (pa). (F) Lateral view of a stage 24 embryo. XPax-2 expression can now also be detected in the furrows of the visceral arches (va), and in both compartments of the developing pronephric kidney, the pronephros (p) and the pronephric duct (pd). XPax-2 expression in the hindbrain (hb) is limited to the region posterior to the otic vesicle. (G) Lateral view of a stage 28 embryo. The proctodaeum (pr) appears as a further tissue expressing XPax-2 transcripts. (H) Lateral view of a stage 32 embryo. Arrowheads indicate XPax-2 expression in the three nephrostomes of the pronephros. Note the appearance of the rectal diverticulum (rd) extending in anterior direction (open arrow) towards the posterior end of the pronephric duct (filled arrow). (I) Lateral view of a stage 36 embryo. The pronephric duct (filled arrow) and the rectal diverticulum (open arrow) are close to joining. Strong expression of XPax-2 is seen in the hindbrain and along the entire spinal cord. (J) Lateral view of a stage 39 embryo. The pronephric duct has fused with the rectal diverticulum (filled arrow). XPax-2 expression is detected in the entire excretory canal up to the point where fusion occurs with the cloaca (open arrow). The brown color in the eye is due to the onset of pigmentation. (K) Close-up view of a stage 39 embryo to illustrate details of XPax-2 expression in the optic stalk (filled arrow), the posterior end of the midbrain (open arrow), the otic vesicle (open arrowhead), and the hindbrain. Note also the extensive coiling of the pronephric tubules and the strong staining associated with the nephrostomes (filled arrowheads). (L) Transversal section through a stage 32 embryo which is cut anterior to the midbrain-hindbrain boundary. XPax-2 expression is confined to the ventral portion of the optic cup (arrowheads) and to furrows of the visceral arch (arrows). (M) Transversal section through a stained stage 32 embryo which is cut at the level of the hindbrain-spinal cord junction. Staining of the pronephroi (arrowheads) and of cells in the ventrolateral neural tube (arrows) is indicated. (N) Horizontal section through the visceral arches of a stage 28 embryo. The plain of section is below the eye. Arrowheads point to the expression in the furrows of the visceral arches. Embryos in shown in (A-K) were photographed uncleared, with the anterior ends of the embryo oriented to the left.
XPax-2 expression was also found to be associated with the development of two sensory organs, the eye and the ear. For the eye, XPax-2 transcripts were initially detected at stage 19 (late neurula) as a faint signal in the region of the future optic stalk and vesicle (Fig. 7C). By stage 21, expression was very prominent, and remained high during later stages as the optic stalk constricts and the optic vesicle invaginates. In transverse sections, XPax-2 transcripts were associated with the ventral region of the optic cup (Fig. 7L). In tadpole stage embryos, XPax-2 expression was gradually down-regulated, first in the optic cup, and then in the optic stalk (Fig. 7K). The onset of XPax-2 transcription in the developing ear correlated with otic placode induction (Fig. 7E). Invagination starts at stage 23, and separation from the epidermis is achieved by stage 28 (Nieuwkoop and Faber, 1956). XPax-2 expression remained associated with the otic vesicle throughout this period. By stage 39, XPax-2 expression was gradually down-regulated in the epithelium of the otic vesicle, but still remained in a patch of dorso-medially located cells (Fig. 7J, K).

Pax-2 expression in the context of visceral arch development has so far not been described. In *Xenopus*, we noticed three prominent stripes of XPax-2 expression which were associated with the visceral arches from stages 24 to 32 (Fig. 7F-H). Visceral arches, referred to as branchial or pharyngeal arches in higher vertebrates, are important for the development of the entire neck region in all vertebrate embryos (Carlson, 1996). The arches originate as bars of mesenchyme sculpted from the sides of the neck by pairs of visceral (or pharyngeal) pouches, which bulge out from the foregut endoderm. Where a visceral pouch approaches the overlying ectodermally-derived epidermis, it displaces the surrounding mesenchymal tissue and induces the formation of a furrow. In *Xenopus*, three visceral furrows have been described (Nieuwkoop and Faber, 1956). Each visceral arch is composed of epithelial layers of both ectodermal and endodermal origin, and contains premuscle mesenchyme of mesoderm origin, and a cranial nerve and a skeletal primordium of neural crest origin. We carried out horizontal sections to determine which of these tissues is associated with XPax-2 expression (Fig. 7N). XPax-2 transcripts were limited to the epidermal layer and highest concentrations were found in the three visceral furrows. Once embryos reached stage 36, the expression ceased (Fig. 7I).

*Expression of XPax-2 in the developing pronephric kidney*

The first morphological indication of the pronephric anlage is seen at stage 21 as a slight thickening of the somatic layer of the intermediate mesoderm below somites 3 and 4 (Nieuwkoop and Faber, 1956). This corresponds exactly
to the first time at which XPax-2 staining could be detected in the developing pronephric kidney. The embryos showed Pax-2 expression in a small extended stripe of tissue below the somites (Fig. 7E). By stage 24, the stripe of cells had extended in posterior direction with the future pronephros and pronephric duct becoming now discernible (Fig. 7F). The subsequent development of the pronephric kidney is characterized by the differentiation of the pronephros (into nephrostomes, tubular canaliculi and the collecting tube), the elongation of the pronephric duct, and the outgrowth of the rectal diverticulum. XPax-2 expression remained associated with the epithelial components of the pronephric kidney during each of these morphogenetic processes (Fig. 7G-K). Transverse sections of stained embryos confirmed expression in the pronephros (Fig. 7M). Formation of the three nephrostomes begins at stage 28 and is completed by stage 34. This process was accompanied by a significant increase in XPax-2 expression as illustrated by three strongly stained spots in the pronephros (Fig. 7H,K). Expression of XPax-2 was also seen throughout the tubular system which was undergoing extensive coiling (Fig. 7K). The excretory canal is formed by fusion of two elongating epithelial components, the pronephric duct and the rectal diverticulum. Posterior elongation of the pronephric duct starts at stage 26, and continues until stage 36/37, when fusion with the rectal diverticulum occurs (Fig. 7H-J). The rectal diverticulum is formed from a bulge of the dorsal wall of the proctodaeum at stage 32 and grows in the anterior direction to make contact with the posterior growing pronephric duct. XPax-2 expression was detected starting from stage 28 in the region of the proctodaeum and became later also associated with the outgrowing rectal diverticulum (Fig. 7G, H). Expression of XPax-2 in the proctodaeum was subsequently down-regulated, first only ventrally and, later, also in dorsal aspects. By stage 39, XPax-2 expression remained in the epithelium of the excretory canal up to the point, where it fuses with the cloaca (compare Fig. 8H-J).

Isolation of cDNAs encoding novel XPax-2 isoforms from the developing pronephric kidney

The isolation of cDNAs encoding novel Pax-2 isoforms from a Xenopus head cDNA library prompted us to investigate alternative splicing of Pax-2 transcripts in the developing pronephric kidney. For this purpose, a cDNA library of XPax-2 cDNAs was prepared from RNA of stage 24 explants enriched for pronephroi (for dissection strategy, see Fig. 11A). For PCR amplification, primers XP-26 and XP-15 were chosen to anneal at the translational start site.
and in the 3'-untranslated region, respectively. Both primers were designed to amplify cDNAs derived from XPax-2a and XPax-2b-encoding transcripts. The resulting cDNA libraries were analyzed systematically with the aim of identifying novel Pax-2 splice variants (see Materials and Methods for details).

Analysis of a total of 140 cDNA clones containing XPax-2 sequences led to the identification of six novel isoforms which were named XPax-2(4) to XPax-2(9). We determined the DNA sequences of XPax-2 cDNA clones representing each novel XPax-2 isoform class and compared them with the previously isolated XPax-2 isoforms (data not shown). Isoforms XPax-2(4), XPax-2(5) and XPax-2(7) were found to be derived from transcripts of the XPax-2a gene, while XPax-2(8) was from the XPax-2b gene. The cDNAs representing isoforms XPax-2(6) and XPax-2(9) were found to be hybrid clones consisting of sequences derived from both XPax-2 genes (see legend to Fig. 8). It is likely that these hybrid clones were generated during the PCR amplification process. Evidence for the existence of XPax-2 mRNA species homologous to isoforms XPax-2(6) and XPax-2(9) in Xenopus embryos and pronephric kidneys will be provided in later (Fig. 10).

In general, the generation of alternatively spliced transcripts can be understood based on the exon/intron organization of the corresponding gene. While the exon-intron structures of the two XPax-2 genes are currently not known, the genomic structure for human Pax-2 has been recently reported (Sanyanusin et al., 1996). Based on sequence comparisons of the Xenopus Pax-2 splice variants with human XPax-2 cDNAs of known exon compositions (Fig. 5; data not shown), it appears that the locations of introns have been conserved during evolution and it is likely that amphibia and humans share the same basic exon organization of their Pax-2 genes. Twelve exons have so far been identified in the human Pax-2 gene. The identification of a novel intervening sequence present in XPax-2b(2) and XPax-2a(4) suggests that Xenopus Pax-2 genes are composed of at least 13 exons. Based on this model, the structural organization of all XPax-2 isoforms isolated in this study has been diagrammed in Fig. 8. It appears that the diversity of XPax-2 isoforms is generated by alternative splicing of exons downstream of the DNA-binding paired domain. Exons 1, 2, 3, 4, 10, 12 and 13 were found to be common to all XPax-2 isoforms, while none appeared to contain sequences encoding exon 6. Finally, all described XPax-2 cDNAs retained the original reading frame despite being subject to extensive alternative splicing events.
Fig. 8. Schematic representation of *Xenopus* Pax-2 isoforms generated by alternative splicing. The structure of the *Xenopus* Pax-2 protein is shown schematically with the corresponding domain organization (PD, paired domain; OP, octapeptide; HD, homeodomain) indicated. The predicted exon structure is based on data from sequence comparisons of *Xenopus* Pax-2 isoforms (see Fig. 5) and on the exon organization of the human Pax-2 gene (Sanyanusin et al., 1996). The positions of the introns are indicated by arrows. Exons are numbered from 1 to 13. The position of sequences in exons 1, 3, 4, 7, 8, 10 and 13, respectively, used to derive primers for RT-PCR are identified by arrows which are labeled with the name of the primer. Below, cDNAs encoding different splice variants of XPax-2 are drawn diagrammatically. Splice variants XPax-2(1), XPax-2(2), and XPax-2(3) represent full-length cDNAs isolated by screening from a *Xenopus* head cDNA library. The cDNAs encoding XPax-2(4) to XPax-2(9) were isolated from a cDNA library generated from pronephric kidney RNA. The exon composition of each splice variant is indicated with numbers. The sequenced cDNAs clones were derived from transcripts of the XPax-2a gene in the case of XPax-2(1), XPax-2(3), XPax-2(4), XPax-2(5) and XPax-2(7) and for XPax-2(2) and XPax-2(8) from the XPax-2b gene. For hybrid clone XPax-2(6), exons 1, 2, 3 and 4 were from XPax-2b and exons 7, 8, 10, 11, 12 and 13 of XPax-2a. For hybrid clone XPax-2(9), exons 1, 2, 3, 4 and 10 were derived from the XPax-2a gene, while exons 12 and 13 originated from the XPax-2b gene.
Alternate use of consensus 3' splice acceptors generates additional diversity among XPax-2 mRNAs

Canonical 3' splice acceptor sequences are characterized by an invariant AG dinucleotide at the -2 and -1 positions of the intron, and are usually preceded by a C or U at the -3 position (Stephens and Schneider, 1992). It has been previously shown that distinct splice forms of Pax mRNAs can be attributed to the use of alternate 3' splice acceptors (Kozmik et al., 1993; Poleev et al., 1995; Vogan et al., 1996). DNA sequence comparisons revealed evidence that similar alternative splicing events occur in XPax-2 transcripts. The N-terminus of isoform XPax-2b(8) differed from XPax-2b(2) by the absence of three nucleotides, CAG, at the junction of exon 1 and 2 (Fig. 9A). The observed deletion is most likely due to alternative splicing to an AG acceptor splice site present in exon 2 as illustrated in Fig. 9. The deduced amino acid sequence of XPax-2b(8) predicts that the proline and glycine residues are replaced by a single arginine residue. This splicing event was not unique to XPax-2b(8), since the exact same CAG trinucleotide was found to be deleted in the cDNA encoding the XPax-2(9) isoform (data not shown). Comparison of the C termini of XPax-2a(7) and XPax-2a(1) revealed that XPax-2a(7) contains an insertion of nine nucleotides located between exon 11 and 12 (Fig. 9B). The insertion probably represents sequences derived from intron 11. A consensus 3' splice site at the boundary with exon 12 could be identified. We suggest that the use of a second 3' splice acceptor flanking the 5' end of the insertion is responsible for the generation of the insertion. The confirmation of this hypothesis will however have to await sequencing of the exon-intron junctions of the XPax-2 genes.

Finally, sequencing of a number of additional XPax-2 cDNA clones revealed the presence of a further consensus 3' splice sequence present in exon 13 (Fig. 9C). Use of this site results in the splicing out of a 19-nucleotide sequence of exon 13. This leads to a shift in the reading frame and gives rise to a Pax-2 isoform with distinct C terminus (Fig. 9D). In summary, our results suggest that differential utilization of consensus 3' splice acceptor sites present in exons 2 and 13, and presumably in intron 11, generates additional diversity of XPax-2 transcripts.
Fig. 9. Novel isoforms of XPax-2 generated by alternate use of 3' splice acceptors. Invariant AG dinucleotides which can serve as consensus 3' splice sites are underlined. Nucleotide sequences altered through use of such alternative 3' splice acceptors are boxed. The new open reading frames are shown below. Amino acids that differ from the original deduced amino acid sequence are indicated with bold letters. The positions of the introns are indicated by arrows and are based on the structure of the human Pax-2 gene (Sanyanusin et al., 1996). (A) XPax-2b(8) contains a three-nucleotide deletion within exon 2. The nucleotide and amino acid sequences of XPax-2b(2) are shown below. The nucleotide sequence deleted in XPax-2b(8) is boxed. (B) XPax-2a(7) contains an insertion of nine nucleotides located between exon 12 and 13. The sequence is presumably derived from intron 11 and has a consensus 3' splice site. For comparison, the equivalent region in XPax-2a(1) is given below. (C) Deletion of nineteen-nucleotide sequence within exon 13 creates a splice variant of XPax-2 with an altered C-terminus. Below, the nucleotide sequence of XPax-2a(1) is shown to outline the nineteen-nucleotide deletion identified in several XPax-2 cDNA clones. (D) Comparison of the deduced amino acid sequences at the extreme C-termini of XPax-2a(1) and XPax-2 splice variants (below) containing the nineteen-nucleotide deletion within exon 13. Asterisks represent stop codons.
Temporal regulation of alternative splicing of XPax-2 transcripts in the early Xenopus embryo

We used RT-PCR to address whether expression of XPax-2 isoforms is regulated during Xenopus embryogenesis. For PCR, several sets of primer pairs were selected based on the proposed exon organization of the XPax-2 genes (Fig. 8). All PCR primers were designed to amplify cDNAs derived from transcripts of both XPax-2 genes. The RT-PCR assay was carried out in conjunction with Southern blotting and hybridization to visualize amplification products encoding XPax-2 sequences. The identity of specific bands was determined in comigration experiments with amplification products of known exon composition (Fig. 10C, D; and data not shown). In some cases, amplification products were also verified by cloning and DNA sequencing (e.g. products marked by asterisks and filled circles in Fig. 10D). For a given XPax-2 isoform, the amplification products of the two pseudoallelic forms are of equal size and can therefore not be distinguished in the assay used here. We will therefore not maintain a distinction between the two pseudoallelic XPax-2 genes in describing the exon composition of the observed amplification products. We also want to stress that RT-PCR was performed "semi-quantitatively" allowing us to assess the presence or absence of specific XPax-2 transcripts rather than determining precise expression levels.

Alternative splicing in the paired domain can dramatically alter the DNA binding affinity of Pax proteins as described for Pax-3, Pax-5 and Pax-6 (Epstein et al., 1994b; Vogan et al., 1996; Zwollo et al., 1997). To determine whether similar alternative splicing events can occur in the paired domain of XPax-2, we selected for RT-PCR primers XP-25 and XP-31 directed against sequences in exon 1 and exon 3, respectively. All cloned XPax-2 cDNAs contain exons 1, 2 and 3 (see Fig. 8), and generate in PCR experiments amplification products of 380 bp (data not shown). RT-PCR experiments using RNA isolated from embryos ranging from unfertilized eggs to stage 34 tadpole embryos consistently resulted in a single amplification product composed of exons 1, 2 and 3 (Fig. 10A). Evidence for maternal transcripts present in unfertilized eggs and in blastula stage embryos was obtained upon overexposure of the blot (data not shown). The vast majority of XPax-2 transcripts were however detected in embryos from stage 12 on with expression levels remaining essential constant throughout the embryonic stages tested (Fig. 10A). These observations are consistent with our in situ hybridization studies, which place the onset of spatially localized XPax-2 transcription to mid-gastrula stages (Fig. 7).
**Fig. 10.** Temporal regulation of alternative splicing of XPax-2 transcripts during early *Xenopus* embryogenesis. Schematic representations of the XPax-2 protein are shown in panels A-D. Conserved sequence motifs (PD, paired domain; OP, octapeptide; HD, partial homeodomain) are outlined, and arrows mark the relative positions of the sequences recognized by the primers used for RT-PCR (see also Fig. 8). Total RNA was extracted from unfertilized eggs (E) and embryos of the indicated stages, and assayed for the expression of XPax-2 splice variants by RT-PCR. Amplification products were separated on agarose gels, transferred onto nylon membranes and hybridized with appropriate radiolabeled XPax-2 probes. The migration positions of size standards are shown on the left of the autoradiographs. On the right, selected amplification products are identified by arrowheads. Their exon composition was determined by comparison with XPax-2 cDNAs of known exon composition (autoradiographs on the right of C and D) and by sequencing of the cloned amplification products. The numbering of exons is based on the model in Fig. 8. (A) Alternative splicing in the paired domain region. Primers XP-25 and XP-31 directed against sequences in exon 1 and 3, respectively, were used for the RT-PCR analysis. A single amplification product with a structural organization consisting of exons 1, 2 and 3 was detected. (B) Alternative splicing in the region of the octapeptide motif. Primers XP-59 and XP-11 are directed against sequences in exon 4 and 7, respectively. The autoradiograph displays the temporal expression profile of alternatively spliced XPax-2 transcripts in the early embryo. (C) Alternative splicing between exons 4 and 10. Primers XP-59 and XP-63 are used for PCR amplification. The autoradiograph on the left displays the spectrum of alternative splice variants in the developing embryo. The autoradiograph on the right compares the amplification products generated with XPax-2 cDNA templates of defined exon composition to those detected in the developing embryo. (D) Alternative splicing in the C-terminal region. Primers XP-9 and XP-24 are directed against sequences in exon 8 and 13, respectively. The autoradiograph on the left displays the spectrum of alternative splice variants in the developing embryo. Amplification products generated by alternate use of 3' splice acceptors present in intron 11 or exon 13 are marked by asterisks or filled circles, respectively. The autoradiograph on the right compares the amplification products generated with XPax-2 cDNA templates of defined exon composition to those detected in the developing embryo. (E) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with ornithine decarboxylase (ODC) specific primer pair ODC-1/ODC-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers.

The next set of PCR primers was chosen to investigate alternative splice events in the region of the octapeptide motif. In particular, we were interested in investigating whether we could detect XPax-2 transcripts containing the predicted exon 6, for which evidence exists in zebrafish, mouse and human (Dressler et al., 1990; Krauss et al., 1991; Sanyanusin et al., 1996). Primers XP-59 and XP-11 were designed to recognize sequences within exon 4 and exon 7, respectively (Fig. 8). RT-PCR experiments allowed us to identify two amplification products (Fig. 10B). The larger amplification product was shown to
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consist of exons 4, 5, and 7, while the smaller one contained exons 4 and 7 (data not shown). Both amplification products could not be detect in pre-gastrula embryos, but were present in embryos from stage 12 on. Together with evidence presented below (Fig. 10D), it appears that XPax-2 transcripts composed of exon 6 are not expressed during early Xenopus embryogenesis.

The exons 4 and 10 are common to all XPax-2 cDNAs described in this study (Fig. 8). We therefore chose primers XP-59 and XP-63 to investigate splicing between these exons. The RT-PCR reactions revealed at least eight distinct XPax-2 bands and for five of the eight amplification products the exon composition was determined (Fig. 10C; and data not shown). None of the amplification products appears to contain exon 6. The majority of the amplification products were detected in gastrula stage and older embryos. Two amplification products, however, were detected also in pre-gastrula embryos. They were found to be composed of exons 4 and 10, and exons 4, 5, and 10, respectively. Evidence for maternal expression of XPax-2 transcripts had also been found with primers annealing to exons 1 and 3, respectively (see text to Fig. 10A). We conclude that maternal transcripts of XPax-2 contain either exons 1, 2, 3, 4 and 10 or exons 1, 2, 3, 4, 5 and 10. Only isoforms XPax-2(7), XPax-2(8) and XPax-2(9) have exon compositions consistent with maternal mRNA expression. Further, XPax-2 isoforms (1) to (6) are expressed exclusively as zygotic mRNAs in the early Xenopus embryo.

The different XPax-2 isoforms diverge mainly in their C-terminal domains indicating that this region is possibly most susceptible to alternative splice events. We therefore selected primers XP-9 and XP-24 to investigate how alternative splicing affects the C-terminal domain between exon 8 and 13 (Fig. 8). This primer pair will detect only expression of strictly zygotic XPax-2 mRNAs. RT-PCR experiments revealed a complex pattern of amplification products ranging in size from 270 to 580 bp (Fig. 10D). Amplification products with exon compositions corresponding to cloned XPax-2 isoforms were readily identified (Fig. 10D, right panel). Two minor bands (identified by asterisks and filled circles in Fig. 10D) were found to be flanking the amplification products containing exons 8, 10, 11, 12 and 13 and exons 8, 10, 12 and 13. We demonstrated by cloning and DNA sequencing that they represent transcripts generated by the alternate use of consensus 3' splice sites present in intron 11 and exon 13, respectively, as described earlier (see Fig. 9B, C). None of the amplification products were detected before mid-gastrula stages indicating that isoforms XPax-2(1) to XPax-2(6) are not expressed as maternal transcripts. Most of the detected amplification products are not subject to significant
regulation of expression levels between gastrula stages and stage 34. The largest amplification product comprising of all exons from 8 to 13 represents an exception, since it is predominantly detected in gastrula and neurula stage embryos. In summary, we have been able to define two classes of XPax-2 mRNAs. A first group consisting of strictly zygotic XPax-2 mRNAs and second one comprised of those transcripts which display, in addition, maternal expression.

Expression of XPax-2 splice variants in the developing pronephric kidney

Isoforms XPax-2(1) to (3) were identified in a cDNA library prepared from heads of stage 28-30 embryos, while XPax-2(4) to (9) were isolated from cDNA libraries of stage 24 pronephric explants. The question therefore arises whether expression of specific XPax-2 isoforms is restricted to the developing pronephric kidney. We employed the RT-PCR approach using now RNA prepared from embryos dissected into heads and explants enriched for pronephroi. Two embryonic stages were chosen for the dissections with the purpose of representing an early (st. 24) as well as an advanced stage (st. 36) of pronephric kidney development. Particular care was taken to assure that pronephric explants were not contaminated with neural tissues (e.g. spinal cord). Head explants contained most of the XPax-2 expressing tissues with the exception of spinal cord and pronephric kidneys (Fig. 11A).

We carried out RT-PCR experiments using primers XP-59 and XP-63 to monitor splicing events between exons 4 and 10, which are found in all described XPax-2 isoforms. Using cDNAs from pronephric kidney explants as templates, we were able to detect at least eight distinct amplification products (Fig. 11B). The exon composition of five bands could be assigned. They represent the most abundant amplification products. Three amplification products remain currently uncharacterized. In most cases, the expression levels of the amplification products did not change between stage 24 and stage 36 pronephroi. Interestingly, the profiles of amplification products observed with cDNAs from pronephric explants were essentially identical to those obtained with cDNAs from heads with the exception of an amplification product composed of exon 4 and 10. This band was found predominantly in older pronephroi, but longer exposures of the blot revealed faint signals in stage 24 pronephroi and in head explants of both embryonic stages (data not shown).
Fig. 11. Alternative splicing of XPax-2 transcripts in the developing pronephric kidney. (A) Outline of the dissection strategy. To illustrate the distribution of XPax-2 expressing tissues, embryos of stage 24 and 36, respectively, stained by in situ hybridization are shown. The dissection planes are indicated with black lines. Heads (h) and explants enriched in pronephric tissue (p) were used for RNA isolation, and subsequent RT-PCR analysis. Head explants contain brain tissues, sensory organs, and visceral arch material. (B) Identification of alternative splice variants using primer pair XP-59/XP-63. Head (h) and pronephric (p) explants were analyzed by RT-PCR. The autoradiograph is shown with the size markers on the left. Amplification products verified by DNA sequencing are identified by arrowheads and their exon compositions are shown. (C) Identification of alternative splice variants using primer pair XP-9/XP-24. (D) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with histone H4 specific primer pair H4-1/H4-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers. The limitations of histone H4 as a control for pronephric RNA preparations are discussed in the result section.
To control for equal amounts of RNA in cDNA synthesis reactions, the expression of commonly used control genes such as ornithine decarboxylase (ODC), elongation factor 1α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or histone H4 was monitored. We found that all of these genes were suitable standards, if the RNA preparation originated from embryonic heads. However, if RNA was obtained from pronephric kidney explants, expression of control genes was either not detectable (ODC, EF-1α, and GAPDH; data not shown) or in the case of histone H4 was found to be at lower levels than expected (Fig. 11D). Similar observations have been reported previously, e.g. for cells of the thyroid (Savonet et al., 1997). We therefore believe that in the absence of a better standard the spectrophotometric measurement of total RNA for pronephric tissues represents the only reliable method of quantification. Taken together, our experiments have so far indicated that splicing of XPax-2 transcripts generates in the pronephric kidney a similar spectrum of isoforms as seen in head explants. Indeed, the expression profiles were found to be indistinguishable to those obtained with cDNAs from whole embryos of the corresponding stages (compare with Fig. 10D).

In a separate set of RT-PCR experiments, we also used primers to sequences in exons 8 and 13 located in the C-terminal domain of XPax-2. As shown in Fig. 10C, amplification products for all XPax-2 isoforms were present in both tissues analyzed. In younger pronephroi, the expression levels of amplification products containing exon 9 were found to be rather low. Higher levels of amplification products containing exon 9 were however detected in older pronephroi, and the presence of XPax-2 transcripts in pronephric kidney was also confirmed by in situ hybridization using a probe consisting solely of exon 9 (data not shown). Finally, we also carried out RT-PCR experiments using primers XP-59 (exon 4) and XP-24 (exon 13). Again, we could not discern any clear difference between XPax-2 isoforms expressed in the head or the pronephric kidney (data not shown). In summary, the evidence present here argue strongly against the expression of a unique complement of XPax-2 splice variants in the developing pronephric kidney. We can however not be excluded that the relative abundance of splice variants might vary during pronephric kidney development.
Results

Induction of XPax-2 transcription in animal cap explants by growth factors

In Xenopus, animal cap cultures represent a convenient way to induce different tissues types in vitro (Asashima, 1994; Dawid, 1994; Kadesh, 1992; Kessler and Melton, 1994; Slack, 1994). For example, the growth factor activin can induce animal cap explants to differentiate in a dose-dependent manner into neural tissue and almost all mesodermal tissues. On the other hand, treatment with basic fibroblast growth factor (bFGF) promotes mainly mesoderm of ventrolateral type. Retinoic acid by itself does not induce mesoderm in animal cap explants, but it can, if added in combination with activin, promote the induction of pronephric tubules at a very high frequency (Moriya et al., 1993; Uochi and Asashima, 1996). We therefore decided to investigate the regulation of XPax-2 expression and alternative splicing under in vitro conditions in animal cap cultures.

Animal caps were excised from stage 8-9 embryos, and treated with either activin, bFGF, retinoic acid, or a combination of activin and retinoic acid. Once control embryos reached stage 28, the caps were analyzed for the expression of XPax-2 by RT-PCR as well as by whole mount in situ hybridization (Fig. 12). Untreated animal caps or caps incubated with retinoic acid alone were unable to activate XPax-2 gene transcription as illustrated in RT-PCR experiments using primers pairs XP-59/XP-63 and XP-9/XP-24, respectively (Fig. 12A, B). Strong induction of XPax-2 transcription was seen in the presence of activin, while bFGF was found to be a much less potent inducer. The profile of amplification products was however very similar for both growth factor and mirrored the situation described earlier for whole embryos (compare Fig. 12A, B with Fig. 10C, D). Interestingly, the inclusion of retinoic acid in the activin-treated cultures neither changed the pattern nor the extent of XPax-2 splicing (Fig. 12A, B). The induction of Pax-2 transcripts in animal cap cultures could also be confirmed by in situ hybridization (Fig. 12D). Animal caps responded to activin by extensive elongation and displayed patches of strong XPax-2 staining. Retinoic acid, in combination with activin, significantly suppressed the elongation process, but still allowed expression of XPax-2 transcripts. Despite the fact that all treatments used here have been described to induce different spectra of tissues in animal caps (Green et al., 1990; Moriya et al., 1993; Uochi and Asashima, 1996), we were unable to detect significant differences in the splicing of XPax-2 transcripts. Based on our in vivo and in vitro experiments, we conclude that splicing of XPax-2 transcripts is not regulated in a tissue-specific manner during early Xenopus embryogenesis.
Fig. 12. Induction of XPax-2 splice variants in animal cap explants. Embryos were cultured until stage 8-9, at which the upper part of the animal hemisphere (animal cap) was removed. The animal caps were treated for 12 hrs at 22°C with either activin (10 ng/ml), retinoic acid (RA, 10 μM), basic FGF (bFGF; 100 ng/ml) or a combination of activin and RA. Control explants were cultured in the absence of any factors. Expression of XPax-2 splice variants was assayed by RT-PCR (panel A-C) and by whole mount in situ hybridization (panel D) once reference embryos reached stage 28. In panels A and B, schematic representations of the XPax-2 protein are shown to illustrate the RT-PCR strategy. Autoradiographs is shown with the size markers on the left. (A) Identification of alternative splice variants using primer pair XP-59/XP-63. (B) Identification of alternative splice variants using primer pair XP-9/XP-24. (C) Control for equal RNA amounts. RT-PCR experiments were carried out in parallel with elongation factor-1α (EF-1α) specific primer pair EF1α-1/EF1α-2. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. M, lane with size markers. (D) Induction of XPax-2 expressing tissues in animal cap explants. Animal caps were incubated either under control conditions or in presence of added factors (activin alone; activin and RA) as described above. Note the absence of XPax-2 transcripts in control animal caps.
5.1.4. Discussion

The simple organization together with its role as an inducer of the mesonephric and metanephric kidney makes the pronephric kidney attractive for studies on the molecular and cellular mechanisms that control kidney organogenesis. In the present study, we report the isolation and characterization of cDNAs for Pax-2, a possible regulator of early kidney organogenesis, from the frog *Xenopus laevis*. We found that XPax-2 gene expression is associated with all epithelial structures of the developing pronephric kidney from the onset of morphogenesis. During this developmental process, XPax-2 transcripts are extensively spliced as illustrated by the isolation of cDNAs encoding multiple novel Pax-2 isoforms from pronephric kidney libraries. An analysis of XPax-2 transcripts from pronephroi and other embryonic tissues revealed that alternative splicing was not regulated in a tissue-specific manner. The implications of our findings will be discussed below.

*Evolution and genomic organization of vertebrate class III Pax genes*

Three genes, Pax-2, Pax-5 and Pax-8, comprise currently the class III Pax gene family in vertebrates (Noll, 1993; Nornes et al., 1996; Walther et al., 1991). Class III Pax genes arose by two independent gene duplications from a common ancestral gene (Noll, 1993). Based on sequence homologies and phylogenetic tree analysis, it is believed that a first duplication event produced Pax-8 and an ancestor gene with properties of both Pax-2 and Pax-5. A second, more recent duplication event generated Pax-2 and Pax-5. Both duplication events apparently occurred in the course of vertebrate evolution after the separation of the lines leading to fish and higher vertebrates (Noll, 1993). Supporting this hypothesis, zebrafish Pax-2 (previously referred to as Pax-B or *pax[zf-b]*) represents the only class III Pax gene identified in fish so far. The complexity of the class III Pax gene family in amphibians was not known up to now. The Pax cDNAs isolated in this study were shown by sequence comparison and expression pattern to be clearly *Xenopus* homologues of mammalian Pax-2 genes. In addition, we have also isolated cDNAs encoding a *Xenopus* Pax-5 homologue (N. Heller and A.W. Brändli; manuscript in preparation). We currently lack evidence for the existence of *Xenopus* Pax-8 homologue. Since the duplication event that generated Pax-2 and Pax-5 is considered to be the evolutionary younger event, the possibility exists that the *Xenopus* genome might also harbor Pax-8 genes. We therefore
believe that the currently known complexity of class III Pax genes was established before the separation of amphibians and higher vertebrates in evolution.

The common evolutionary origin of the class III Pax genes is also reflected in their genomic organization. All the known intron positions are strictly conserved between human Pax-2, Pax-5 and Pax-8 (Busslinger et al., 1996; Kozmik et al., 1993; Poleev et al., 1995; Sanyanusin et al., 1996). Although the genomic organization of the *Xenopus* Pax-2 genes are currently not known, the evidence from our studies on alternative splicing of XPax-2 transcripts are consistent with this notion. For example, sequence comparisons revealed that XPax-2a(1) and XPax-2a(3) represent *Xenopus* homologues of previously reported human Pax-2 splice variants (Fig. 5B). Furthermore, all XPax-2 isoforms reported here can be explained using the exon organization of the human Pax-2 gene as a paradigm (Fig. 8).

Two cDNAs, XPax-2b(2) and XPax-2a(4), are generated by inclusion of a novel intervening sequence (Fig. 5B, 8). Interestingly, the human Pax-8 gene contains a homologous sequence encoded by an alternatively spliced exon (identified as exon 8 (Poleev et al., 1995) or exon 9 (Kozmik et al., 1993), respectively). It is therefore likely that the novel XPax-2 intervening sequence is also encoded by an exon, which would represent a novel vertebrate Pax-2 exon. Based on this observation, we propose that the coding sequences of the *Xenopus* Pax-2 can be composed of up to 13 exons (Fig. 8). We have so far been able to obtain nucleotide sequence information on all exons, but exon 6. Isolation of genomic clones of XPax-2 should resolve this remaining open question.

**Pax genes in visceral arch development**

Analysis of XPax-2 expression during *Xenopus* embryogenesis revealed that transcripts were associated with the developing nervous system, sensory organs and the excretory system (Fig. 7). This expression pattern is essentially identical to that reported for Pax-2 homologues in zebrafish (Krauss et al., 1991; Mikkola et al., 1992; Püschel et al., 1992) and mice (Dressler et al., 1990; Nornes et al., 1990; Püschel et al., 1992) and indicates that the mechanisms controlling tissue-specific regulation of Pax-2 gene expression have been conserved during vertebrate evolution. In addition, we identified Pax-2 expression in the ectodermally derived furrows of the visceral arches (Fig. 7N). This novel site of Pax-2 expression has possibly been overseen in previous studies of zebrafish and mouse embryos.
Results

What could be the role of Pax-2 in visceral arch development of the vertebrate embryo? In water-living embryos of lower vertebrates, the furrows and pouches of the visceral arches fuse to form the visceral clefts or gill slits. It is conceivable that Pax-2 could have a role in this process by regulating the fusion process. Other morphogenetic processes leading to fusion processes, such as optic fissure and neural tube closure, have been shown in the past to depend on Pax-2 gene function (Favor et al., 1996; Torres et al., 1996). In the mammalian embryo, only the first furrow gives rise to a recognizable structure (Carlson, 1996). It persists as the outer ear, while the other furrows are overgrown. The tympanic membrane (or ear drum) is formed where the first furrow meets the first pharyngeal pouch. By extension, Pax-2 could be necessary for some aspects of ear development in the mammalian embryo. Recent studies of mouse mutants have shown that Pax-2 is required for the development of the auditory system in the inner ear (Torres et al., 1996). Whether a lack of Pax-2 expression also affects the morphogenesis of the outer ear or the formation of the tympanic membrane remains to be established.

Expression of XPax-2 in the developing pronephric kidney

We have demonstrated that XPax-2 expression is associated with developing pronephric kidney (Fig. 7). How does its expression correlate with the major events of organogenesis: induction, determination, morphogenesis, and differentiation? The time point at which pronephric kidney induction occurs in the amphibian embryo is unknown, but the prospective pronephric area has been mapped in gastrulating embryos to a region of the marginal zone ventrolateral to the blastopore (Pasteels, 1942). Determination occurs by the midneurula stage, when the prospective pronephric area will form pronephric tubules, even when transplanted to heterotopic sites (Fales, 1935). As morphologically distinguishable structure, the pronephric anlage becomes first apparent with the completion of neurulation. In Xenopus embryos, it appears as a homogenous bulge in the mesoderm directly ventral to the somites at approximately embryonic stage 21, which correlates precisely with the first detectable expression of XPax-2 (Fig. 7E). The timing of XPax-2 expression makes it therefore rather unlikely that XPax-2 has a central role in the determination process, but rather suggests a role in pronephric kidney morphogenesis and/or differentiation. This view is supported by studies in zebrafish and mice deficient for Pax-2, where the earliest defects in the excretory system are associated with the elongation of the pronephric duct (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995).
A poorly understood aspect of pronephric kidney development is the contribution of the rectal diverticulum to the formation of the excretory canal. In the tailbud stage Xenopus embryo, a pair of rectal diverticuli form by evagination from the dorsal wall of the proctodaeum. They elongate in the anterior direction to fuse with the posterior growing pronephric ducts. XPax-2 was found to be expressed in both the proctodaeum and the extending rectal diverticuli, demonstrating unequivocally their contribution to the establishment of the excretory canal (Fig. 7G-J). To our knowledge, XPax-2 represents the first molecular marker found to be expressed in the rectal diverticuli.

**Alternative splicing of class III Pax gene transcripts**

The cloning of Pax-2 cDNAs from zebrafish (Krauss et al., 1991), mouse (Dressier et al., 1990) and human (Eccles et al., 1992; Sanyanusin et al., 1996; Ward et al., 1994) had led to the identification of three Pax-2 splice isoforms. In Xenopus, we have isolated through screening of head and pronephric kidney cDNA libraries clones for nine distinct isoforms (Fig. 8). Two of these isoforms, XPax-2a(1) and XPax-2a(3), were homologous to previously identified mammalian isoforms. Thus, alternative splicing of Pax-2 transcripts apparently generates at least ten distinct vertebrate Pax-2 isoforms. We believe that the number of expressed splice variants generated in vivo is in fact even higher. For one, the exon compositions of a number of amplification products are still unknown (see Fig. 10B, C). For the other, we have provided evidence that the diversity of Pax-2 transcripts is further increased by through alternate use of consensus 3' splice acceptors (Fig. 9). These alternative splicing events can either lead to small deletions or insertions of one to three amino acids residues, while still preserving the original reading frame, as observed with XPax-2b(8) and XPax-2a(7). In the case where exon 13 is affected, splicing can even generate Pax-2 isoform with an alternative C-terminus (Fig. 9C, D). A Pax-2 isoform with an alternative C-terminal domain has been previously also reported for human Pax-2 (Ward et al., 1994). Transcripts of the related Pax-5 and Pax-8 genes are also modified by alternative splicing. For Pax-5, transcription from two distinct promotors results in splicing of two alternative 5' exons, exon 1a and exon 1b, to the common coding sequence (Busslinger et al., 1996). In addition, three novel splice forms have been recently found to be expressed during B-cell development (Zwollo et al., 1997). For the Pax-8, a total of seven splice variants have been identified (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). Alternative splicing of class III Pax gene transcripts is therefore not uncommon, but our studies have revealed that
XPax-2 transcripts are subjected to a degree of alternative splicing that had previously not been anticipated.

Functional implications of Pax-2 splicing

Transcripts that either contain or lack particular exons may have entirely different functions at the protein level (Foulkes and Sassone-Corsi, 1992; McKeown, 1992). Pax proteins are thought to function as transcription factors by being able to bind to specific DNA elements of target genes and to act as transcriptional activators. Alternative splicing has been found to affect both aspects of Pax protein function. Introduction of an additional exon into the paired domain of Pax-6 modifies the structure and DNA-binding properties of the protein (Epstein et al., 1994b), while deletion of an exon in the Pax-5 paired domain abolishes DNA binding completely (Zwollo et al., 1997). An alternative mechanism has been described for Pax-3 and Pax-7, where alternate use of consensus 3’ splice sites results in the removal of a single glutamine residue generating isoforms with increased affinity to target DNA sequences (Vogan et al., 1996). Structural motifs mediating transactivation are located in the C-terminal domain of Pax proteins, a region that is preferentially modified by alternative splicing. For Pax-8, several isoforms carrying alternative C termini have been described to harbor distinct transactivation abilities (Dehbi and Pelletier, 1996; Kozmik et al., 1993; Poleev et al., 1995). Similar results were recently reported for isoforms of Pax-9 (Nornes et al., 1996).

How does alternative splicing modulate the function of Pax-2 isoforms? Due to the limited number of Pax-2 variants described so far, the consequences of Pax-2 splicing on protein function are poorly understood. Following predictions can however be made. Exons 1 to 4 of vertebrate Pax-2 transcripts encode the paired domain, which is sufficient to mediate DNA binding (Epstein et al., 1994a; Fickenscher et al., 1993). Since these exons are also present in all XPax-2 isoforms, it is very likely that all will be able to bind target sequences. The 3-nucleotide deletion found in exon 2 of XPax-2(8) and XPax-2(9) might, however, modify the DNA binding characteristics as has been described recently for Pax-3 and Pax-7 (Vogan et al., 1996). The highly conserved octapeptide sequence is encoded by exon 5 and appears to act as a repressor of the activation potential (Lechner and Dressler, 1996). Interestingly, XPax-2(5), XPax-2(6) and XPax-2(9) represent naturally occurring variants lacking exon 5 and could therefore be more potent transactivators in vivo. Comparable transactivation properties were reported for the two Pax-2 isoforms which differ by containing or lacking exon 6 (Dehbi et al., 1996; Kozmik et al., 1993;
Lechner and Dressler, 1996). We were unable to detect XPax-2 transcripts carrying sequences related to exon 6 (Fig. 10B), although such transcripts have been described in zebrafish, mouse and humans (Dressler et al., 1990; Eccles et al., 1992; Krauss et al., 1992; Sanyanusin et al., 1996). It is possible that the expression of exon 6 containing XPax-2 transcripts is developmentally regulated in *Xenopus*, and occurs only in advanced embryos or in adult animals which were not analyzed in this study. Alternatively, mutations inactivating correct splicing of exon 6 might have occurred during the evolution of *Xenopus laevis*. In the light of the apparent lack of functional significance of exon 6, such mutations might not pose a selective disadvantage for carriers of the mutations. Proof whether such mutations occurred indeed will have to await cloning of the XPax-2 genes. The role of the partial homeodomain motif, found in all class III Pax genes, has so far not been determined. XPax-2(7), XPax-2(8) and XPax-2(9) were found to lack the homeodomain encoded by exon 7 and might therefore represent useful tools to resolve this question. Finally, alternative splice events in the C-terminal domain of Pax-2 can generate isoforms which *in vitro* confer differential regulation of the human WT-1 promotor (McConnell et al., 1997). The transcriptional competence appears also to be regulated by a regulatory module composed of activating and inhibitory sequences located at the C-terminal end of all class III Pax proteins (Dörfler and Busslinger, 1996). For XPax-2 isoforms, the presence or absence of exon 11 represents the only splice event observed in this region. Inclusion of exon 11 would extend the transactivation domain defined by Dörfler et al. (1996) and could modulate the transactivating properties of the described regulatory module. In summary, the nine distinct Pax-2 cDNAs isolated in this study can be considered as an unique collection of variants generated by *in vivo* "deletion mutagenesis". Testing their DNA binding and transactivation action properties will complement previous structure-function studies carried out with *in vitro* mutagenized Pax-2 proteins (Dörfler and Busslinger, 1996; Lechner and Dressler, 1996).

**Developmental regulation of Pax-2 splicing**

For transcription factors, developmental regulation of alternative splicing is a widespread phenomenon, but the biological consequences are understood in only a very few cases (López, 1995). A number of recent studies have demonstrated that alternative spliced products of Pax-8 are not only temporally, but also spatially regulated during embryogenesis (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995). Wo previous studies using nuclease protection assays had failed to detect any temporal nor spatial regulation of Pax-2 splicing...
Results

(Dressler and Douglass, 1992; Fickenscher et al., 1993). The probes used in these studies span only a limited amount of the coding sequence and were chosen with the intention to discriminate between the two only Pax-2 splice variants known at the time. Many of the limitations inherent to nuclease protection assays have been overcome by the PCR-based methodology used in this study. In particular, we are now able to display the entire spectrum of splice variants within the experimental boundaries defined by the PCR primers used. We therefore reinvestigated comprehensively the question of developmental regulation of Pax-2 splicing by analysis of whole embryos, explants of enriched pronephric tissues and in vitro generated tissues. Our studies on whole embryos clearly established that alternative splicing of XPax-2 transcripts is under temporal control during embryogenesis (Fig. 10). A limited number of XPax-2 transcripts represented by XPax-2(7), XPax-2(8) and XPax-2(9) were found to be expressed already as maternal transcripts in the pregastrula embryo. These maternal isoforms are characterized by the lack exons 6, 7, 8, and 9. They however still contain the minimal structural elements to mediate DNA binding and transactivation properties. The functional significance of maternal Pax-2 transcripts is unclear, especially since Pax-2 function is not necessary for gastrulation (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995; Torres et al., 1996). The completion of gastrulation is marked by a sudden burst of XPax-2 gene expression. At this time point, the complete range of XPax-2 splice variants can already be detected (Fig. 10). In situ hybridization identifies the future midbrain-hindbrain boundary as the only source of XPax-2 gene expression at this developmental stage (Fig. 7A, B). This observation suggests that a single tissue can generate the full spectrum of Pax-2 isoforms. We then focused our attention to the developing pronephric kidney. Explants enriched for pronephroi as well as in vitro generated pronephric tissues were examined to determine the extent of XPax-2 isoform expression in this organ (Fig. 11, 12). The results presented in this study clearly demonstrate that pronephric kidneys express the complete set of XPax-2 isoforms and that there is little variation in the relative abundance of transcripts during pronephric kidney organogenesis. A similar apparent lack of tissue-specific regulation of alternative splicing is also seen with Pax-3, Pax-6 and Pax-7 (Epstein et al., 1994b; Vogan et al., 1996). The analysis was however limited to single splice events as in previous studies addressing Pax-2 splicing (Dahl et al., 1997; Dressler and Douglass, 1992; Fickenscher et al., 1993). In the present study, we demonstrate now for pronephroi that the absence of tissue-specificity extents to all splice events for Pax-2. It is therefore rather unlikely that certain
isoforms of Pax-2 could have tissue- or organ-specific functions. Our findings rather suggest that alternative splicing may simply serve to increase the functional diversity of Pax-2 genes during embryogenesis. Further characterization of the Pax-2 splice variants will now allow us to design experiments in which the expression levels of different isoforms in *Xenopus* embryos and in growth factor-treated animal cap cultures will be altered. Ultimately, we hope to achieve a better understanding of how the different Pax-2 isoforms function in establishing and controlling the inductive cascades of events necessary for kidney organogenesis.
5.1.5. Materials and Methods

*Growth factors and retinoic acid*

Recombinant *Xenopus* basic FGF (bFGF) was prepared from *E. coli* strain XF140 using a T7 expression system essentially as described (Kimelman et al., 1988). An additional ion-exchange chromatography step was added to the protocol. In brief, the cleared bacterial lysate was adjusted to 0.1 M sodium phosphate, pH 6.0, and loaded onto a 80-ml CM Sephadex C25 column (Pharmacia Biotech). The column was washed with 0.1 M sodium phosphate, pH 6.0, and then with 0.1 M sodium phosphate, 0.1 M NaCl, pH 6.0. The bound protein was eluted with 0.1 M sodium phosphate, 0.4 M NaCl, pH 6.0. Peak fractions were collected, pooled, and applied to a 5-ml HiTrap Heparin column (Pharmacia Biotech). The column was washed first with 0.6 M NaCl, 10 mM Hepes, pH 7.2 and then with 1 M NaCl, 10 mM Hepes, pH 7.2. The bound protein was eluted with 2 M NaCl, 10 mM Hepes, pH 7.2. The eluates were concentrated to 10 mg/ml and the buffer was exchanged with 10 M Tris-HCl, pH 7.0 using Centriprep-10 concentration devices (Millipore). The purity of each preparation was assessed by SDS-polyacrylamide electrophoresis. Only preparations consisting of a single 17 kDa band were used. For animal cap experiments, purified *Xenopus* bFGF preparation were diluted with 0.5x MMR (1x MMR: 100 mM NaCl; 2 mM KCl; 2 mM CaCl$_2$; 1 mM MgSO$_4$; 1 mM EDTA; 5 mM Hepes, pH 7.4) containing 1 mg/ml BSA to a concentration of 10 μg/ml and stored at -80°C. Each preparation was also tested in animal cap assays for bioactivity (Kimelman et al., 1988). *Xenopus* bFGF was usually used at 100 ng/ml.

Recombinant human activin A was kindly provided by Kristin Verschueren and Danny Huylebroeck (Laboratory of Molecular Biology, University of Leuven, Leuven, Belgium). Activin A was dissolved in 0.5x MMR containing 1 mg/ml BSA to a concentration of 1 μg/ml and stored at -80°C. The bioactivity of the activin A preparation was tested by serial dilution using the animal cap assay. A working concentration of 5-10 ng/ml was found to give a robust induction as determined by massive elongation of the animal cap explants.

*All-trans* retinoic acid (Sigma, R-2625) was dissolved in dimethylsulfoxide (DMSO) at 10 mM under dim light. Aliquots were stored at -80°C. Retinoic acid was used at a concentration of 10 μM (Moriya et al., 1993).
Embryo collection and microdissections

Pigmented *Xenopus laevis* frogs were purchased from African Reptile Park (Tokai, South Africa), and albinos from Dr. Charles Thiébaud (Station de zoologie expérimentale, Université de Genève, Chêne-Bougeries, Switzerland). *Xenopus* embryos were obtained by *in vitro* fertilization as previously described (Brändli and Kirschner, 1995; Newport and Kirschner, 1982). Embryos were staged according to Nieuwkoop and Faber (1956).

All embryo dissections were performed in 1x MMR. Embryos were raised to stage 24 and 36 for dissection of heads and pronephric explants. Embryos were anesthetized in 1x MMR containing 0.1% tricaine (ethyl-3-aminobenzoate methansulfonic acid; Sigma, A-5040). Dissections were done using a microdissecting knife and watch-makers forceps (Fine Science Tools). First, the heads were cut immediately posterior to the visceral arches. Then, explants enriched for pronephroi ('pronephric explants') were obtained by placing a horizontal cut just below the somites to separate dorsal structures (notochord, somites, and neural tube) from ventrolateral tissues (pronephroi, ventral mesoderm, endoderm). Heads and pronephric explants were frozen in liquid nitrogen for later isolation of RNA. See also Fig. 11A, for an outline of the dissection strategy.

Animal cap assays

Animal caps were isolated from stage 8-9 embryos using watch-makers forceps (Dumont No. 5; Fine Science Tools), eye-brow knives and hair loops. The area of dissection was limited to the upper portion of the animal hemisphere, corresponding to roughly one fourth of the embryo. Care was taken to avoid any marginal zone cells, or large, vegetal cells. Explants were cultured in 0.5x MMR containing 1 mg/ml bovine serum albumin (Sigma, A-3350). Purified growth factors (*Xenopus* bFGF or human activin A) and retinoic acid were added at the desired concentrations. Animal caps were incubated in the dark at 22°C for 12 hrs, after which they were rinsed and transferred to 0.5x MMR/BSA. Animal caps were cultured until control embryos reached stage 28. For further analysis, animal caps were either frozen in liquid nitrogen for later isolation of RNA or fixed for whole mount *in situ* hybridization.

Isolation of *XPax-2* cDNAs by library screening

Partial DNA sequences of *Xenopus* Pax-2 were isolated by the reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers.
The following degenerate primer pair was used (see pp. 72/3 for sequences): XP-1 coding for the target peptide RQRIVE; XP-2 coding for G(V/I)CDN(D/E). EcoRI and XbaI restriction sites were added to the sense and antisense primers, respectively, for convenient ligation. Complementary DNAs (cDNAs) were synthesized from 5 μg of total RNA isolated from stage 21, 24 and 30 Xenopus embryos, respectively, using 500 units of Molony murine leukemia virus reverse transcriptase (Superscript II, GIBCO BRL) and 200 pmol of degenerate primer XP-2 in a total volume of 50 μl. One-twentieth of the cDNA preparation was used as a template for amplification of Pax sequences. PCR was performed in a final volume of 50 μl containing 10 mM Tris pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTPs, 100 pmol of each primer (XP-1 and XP-2), and 2.5 units of Taq DNA polymerase (Perkin-Elmer). The amplification parameters were 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 35 cycles followed by 10 min at 72°C. One-third of the amplification products were size fractionated on a 4% NuSieve 3:1 agarose (FMC BioProducts) gel. Amplification products of the expected size, ~280 bp, were excised, and purified using GENECLEAN SPIN kit (BIO 101 Inc.). The recovered amplification products were digested with EcoRI and XbaI, repurified and cloned into pBluescript II SK+ vectors (Stratagene). Competent E. coli DH5α bacteria (MAX efficiency, GIBCO BRL) were used for transformation. The three cDNAs libraries (st. 21, 24, and 30) were screened for clones with sequences homologous to Pax-2. Altogether 88 cDNA clones were analyzed by restriction fragment mapping and DNA sequencing as described (Brändli and Kirschner, 1995). DNA sequences were determined from alkaline lysis miniprep DNA using the dideoxy chain termination method (Sequenase version 2.0, Amersham).

Amplification products encoding XPax-2a and XPax-2b were used to screen a Xenopus stage 28-30 head cDNA library (Hemmati-Brivanlou et al., 1991) ligated into an excisable λZAPII vector (kindly provided by Richard Harland, University of California at Berkeley). Fragments were radiolabeled with [³²P]dCTP (Amersham) by random priming (Oligolabelling Kit, Pharmacia Biotech), and purified on NUCTRAP push columns (Stratagene). Library screening was carried out under conditions of high stringency (Maniatis et al., 1989). Positive clones were purified, and converted to Bluescript plasmids using the Rapid Excision Kit (Stratagene) according to the manufacturer's recommendations. Plasmids were analyzed by restriction fragment mapping and DNA sequencing. Three cDNA classes were identified, and the longest cDNAs [2.0 kb for XPax-2a(1), 3.0 kb for XPax-2a(3), and 2.7 kb for XPax-
2b(2)] were sequenced. The EMBL Nucleotide Sequence Database accession numbers can be found in the next section.

**Isolation of XPax-2 cDNAs by PCR amplification**

Sequences common to both XPax-2 genes were chosen to synthesize oligonucleotide primers with the aim of amplifying by PCR the entire open reading frame of XPax-2 cDNAs. Primer XP-26 corresponds to nucleotides in exon 1 starting at the first translational start site. Primer XP-15 is complementary to sequences in the 3'-untranslated region of exon 13. Sequences of primers are listed on pp. 72/3. Nucleotide sequences encoding EcoR I and Xba I restriction sites were added to the 5'-ends of XP-26 and XP-15, respectively. *Xenopus* explants enriched for pronephroi were microdissected from stage 24 embryos (see 4.2. and Fig. 11A) and used for isolation of total RNA (see 4.6.). First strand cDNA was generated essential as described (see 4.4.) using 0.2 pmol of the antisense primer XP-15 and 10 μg of total RNA. PCR amplification was carried out with the Expand High Fidelity PCR system (Boehringer Mannheim) using one-fortieth of the cDNA preparation as a template. The resulting amplification products were separated by electrophoresis on a 1% agarose gel. A section of the gel containing amplification products ranging between 0.7 and 1.8 kb in size was isolated, and cut into eight slices containing products of increasing size. For each slice, DNA was purified, enriched by a second round of PCR amplification and cloned into the pBluescript II SK (+). From the eight PCR-generated cDNA libraries, a total of 140 cDNA clones were selected and further analyzed by PCR. PCR reactions using different sets of primer pairs were performed to determine the exon composition of each cDNA. Clones representing novel splice variants of XPax-2 were confirmed by sequencing the entire cDNA.

The nucleotide sequences of all XPax-2 cDNAs were submitted to the EMBL Nucleotide Sequence Database, and the accession numbers are as follows: Y10119 for XPax-2a(1), Y10120 for XPax-2b(2), Y10121 for XPax-2a(3), Y10122 for XPax-2a(4), Y10123 for XPax-2a(5), AJ000666 for XPax-2(6), AJ000667 for XPax-2a(7), AJ000668 for XPax-2b(8), and AJ000669 for XPax-2(9).

**RNA isolation**

Total RNA was isolated from unfertilized eggs, embryos and explants using the guanidinium-isothiocyanate method (TRIzol Reagent; GIBCO BRL).
The RNA was precipitated with 1/10 vol. of 0.5 M sodium acetate, pH 5.2 and 2 vol. of 100% ethanol. Precipitated RNA was washed with 70% ethanol and resuspended in an appropriate volume of water. RNA concentrations were determined spectrophotometrically.

**RT-PCR and Southern blot analysis**

For amplification of cDNAs encoding alternatively spliced Pax-2 transcripts, total RNA was extracted from unfertilized eggs, embryos of different developmental stages, and from tissue samples (heads, pronephric explants, and animal caps) as described above. A mixture of XPax-2 antisense primers (XP-11, XP-24, XP-31, XP-63; 0.2 pmol each) was used to synthesize first-strand cDNAs from 10 µg of total RNA as described in the above section. Furthermore, antisense primers (EF1α-2, GAPDH-2, H4-2 and ODC-2; 0.2 pmol each) complementary to *Xenopus* elongation factor 1α (EF1α), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), histone H4 and ornithine decarboxylase (ODC), mRNAs were included in the cDNA synthesis reaction to control for equal mRNA concentrations. One-fortieth of the cDNA preparation was used as template for subsequent amplification by PCR. Where cloned XPax-2 cDNAs were used as templates for PCR, purified plasmids were added as templates at 1 ng per 50-µl reaction. Each primer was added at 50 pmol. The amplification parameters were 94°C for 1 min, 48°C for 40 sec, and 72°C for 35 cycles followed by 10 min at 72°C. One-third of the amplification product was size fractionated on either a 4% or 6% NuSieve 3:1 agarose gel.

Agarose gels were subjected to Southern blotting and hybridized with radiolabeled XPax-2-specific probes for unequivocal identification of amplification products encoding XPax-2 sequences. In brief, amplification products were capillary blotted onto nylon membranes (HYBOND-N+, Amersham) in 0.4 N NaOH and crosslinked to the membrane by UV-irradiation (UV Stratalinker 1800, Stratagene). The filters were hybridized for 18 h in hybridization buffer (5x SSPE, 5x Denhardt's solution, 0.5% SDS, 20 µg/ml denatured fragmented Salmon sperm DNA) at 65°C with a radiolabeled probe. After hybridization, the filters were washed twice for 10 min in 2x SSPE, 0.1% (w/v) SDS at room temperature and once for 10 min in 0.2x SSPE, 0.1% (w/v) SDS at 65°C.

DNA for the synthesis of radiolabeled probes to detect splicing in the paired domain region (probe 1; primers: XP-25 and XP-31) or the octapeptide region (probe 2; primers: XP-59 and XP-11) were generated by PCR with XPax-2a(1) cDNA (1 ng per 50-µl reaction) as template DNA. The purified
amplification products were radiolabeled by random priming as described (see 4.4.). In all other cases, probe 3 directed against both allelic forms of exon 10 was used for hybridization. Radiolabeled probe 3 DNA was generated by PCR using the primers XP-62 and XP-63 and XPax-2a (1) and XPax-2b(2) cDNAs as templates. PCR was performed in a final volume of 30 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 16 µM of dATPs, dGTPs and dTTPs, 5 pmol of [³²P]dCTP, 1 pmol of each primer (XP-62 and XP-63), and 1 unit of Taq DNA polymerase (Perkin-Elmer). The amplification parameters were 94°C for 45 sec, 48°C for 2 min, and 72°C for 1 min for 10 cycles. The radiolabeled probe 3 was purified using a G50 Sephadex column (Boehringer Mannheim) according to the manufacturer's recommendations.

Amplification products were verified by DNA sequencing as follows. After separation by agarose gel electrophoresis, bands were excised from the gel, purified and subcloned into the PCR2.1 vector (Original TA Cloning Kit, Invitrogen) according to the manufacturer's recommendations.

**Primers**

The following primers were used for cDNA synthesis and PCR amplification:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1α1</td>
<td>5'-CAG ATT GGT GCT GGA TAT GC-3'</td>
<td>sense</td>
</tr>
<tr>
<td>EF1α2</td>
<td>5'- ACT GCC TTT ATG ACG CCT AG -3'</td>
<td>antisense</td>
</tr>
<tr>
<td>GAPDH-1</td>
<td>5'-ATT CCC GCC TCA ACT GT-3'</td>
<td>sense</td>
</tr>
<tr>
<td>GAPDH-2</td>
<td>5'-AGG ATG GGC GAC TCT GG-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>H4-1</td>
<td>5'-ATA ACA TCC AGG GCA TCA CC-3'</td>
<td>sense</td>
</tr>
<tr>
<td>H4-2</td>
<td>5'-ACA TCC ATA GCG GTG ACG G-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>ODC-1</td>
<td>5'-GTG AAT GAT GGA GTG TAT GGA TC-3'</td>
<td>sense</td>
</tr>
<tr>
<td>ODC-2</td>
<td>5'-TCC ATT CCG CTC TCC TGA GCA C-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>XP-1</td>
<td>5'-(C/A)GI CA(A/G) (C/A)GI AT(A/C/T) GTI GA-3'</td>
<td>sense</td>
</tr>
<tr>
<td>XP-2</td>
<td>5'-TC(A/G) TT(A/G) TC(A/G) CIA A(C/T)I CC-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>XP-9</td>
<td>5'-GAC CTA GGA AGC AAT GT-3'</td>
<td>sense</td>
</tr>
<tr>
<td>XP-11</td>
<td>5'-AAA CTT TCA ACA CTG CT-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>XP-15</td>
<td>5'-GGT ACA ATA TGG AGG CC-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>XP-24</td>
<td>5'-GCC CCT GGA TGT GGC AC-3'</td>
<td>antisense</td>
</tr>
<tr>
<td>XP-25</td>
<td>5'-GGA TAT GGA CTG CAA GG-3'</td>
<td>sense</td>
</tr>
<tr>
<td>XP-26</td>
<td>5'-ATG GAT ATG CAC TGC AAG G-3'</td>
<td>sense</td>
</tr>
<tr>
<td>XP-31</td>
<td>5'-TCA TTC TCG GAT TCC CC-3'</td>
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<td>XP-59</td>
<td>5'-CAC (A/G)CC C/TGG GCA TCA TC-3'</td>
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<tr>
<td>XP-62</td>
<td>5'-CGA GAT ATG TCA AGC AC-3'</td>
<td>sense</td>
</tr>
<tr>
<td>XP-63</td>
<td>5'-CCA GGT ACC ATT CCA GC-3'</td>
<td>antisense</td>
</tr>
</tbody>
</table>
Whole mount in situ hybridization and probe synthesis

Whole mount in situ hybridization was performed using digoxigenin-labeled RNA probes (Harland, 1991). The standard protocol was modified by replacing methanol with ethanol, and by omitting the RNase digestion step. Fixed animal cap explants were bleached in 70% ethanol/10% H2O2 prior to in situ hybridization.

The following plasmids were constructed for probe synthesis: (a) plasmid Bs4A.3, containing the entire coding sequence (nucleotides 19-1203) of XPax-2a(1); (b) plasmid Bs4B.4, containing the C-terminal domain (nucleotides 799-1203) of XPax-2a(1); (c) plasmid Bs10B.4', containing the C-terminal domain (nucleotides 900-1403) of XPax-2a(3); (d) plasmid Bs7B.5, containing the C-terminal domain (nucleotides 850-1464) of XPax-2b(2); (e) pBs7C.1, containing exon 9 (nucleotides 988-1197) of XPax-2b(2); and (f) pBs10C.1, containing exon 11 (nucleotides 1140-1238) of XPax-2a(3). The plasmids were generated using PCR-based subcloning procedures. The PCR primers were designed with EcoR I (for sense primers) and Xba I (for antisense primers) restriction sites for convenient ligation. Amplification products were ligated into Bluescript II SK+ vector.

Digoxigenin-labeled antisense RNA probes were synthesized with T3 RNA polymerase (MBI Fermentas) from EcoR I linearized plasmids. For control purposes, sense probes were synthesized with T7 RNA polymerase from Xba I linearized plasmids. The in vitro synthesized probes had the following approximate sizes: 1.2 kb for plasmid Bs4A.3; 0.4 kb for plasmid Bs4B.4; 0.5 kb for plasmid Bs10B.4'; 0.6 kb for plasmid Bs7B.5; 0.2 kb for plasmid Bs7C.1; and 0.1 kb for plasmid Bs10C.1.

Stained embryos were visualized and photographed using a Zeiss STEMI 2000-C stereoscopic microscope equipped with a Zeiss MC80 camera using either Kodak Ektachrome 64T or Fujichrome 64T professional slide film. Color slides were scanned using a slide scanner (Polaroid SprintScan 35), and processed using Adobe Photoshop 3.0 and Canvas 3.0 software.

Tissue sectioning

Embryos stained in whole mount were fixed for 1-2 h in MEMFA (Harland, 1991), washed with PBS and embedded in 3% low-melting agarose (SeaPlaque, FMC BioProducts). 50-100 μm sections were cut with a vibrating blade microtome (Leica VT1000M). Photographs were taken and processed exactly as described above.
Acknowledgements

We would like to thank Richard Harland for providing the *Xenopus* stage 28 head cDNA library; Chi Tran for an introduction to cDNA library screening and critical reading of the manuscript; Ursel Müller and Herbert Steinbeisser for advice in sectioning using a vibrating blade microtome; Kristin Verschueren and Danny Huylebroeck for providing recombinant activin A; and David Turner suggesting many helpful improvements to the whole-mount *in situ* hybridization protocol. A.W.B. is a recipient of a Career Development Award of the Swiss National Science Foundation (START #31-38807.93). This work was supported by a grant from the Swiss National Science Foundation (#31-40475.94) to A.W.B.
5.2. *Xenopus* Pax-2/5/8 orthologues: novel insights into Pax gene evolution and identification of Pax-8 as the earliest marker for otic and pronephric cell lineages

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5.2.1. Abstract

Pax genes are a family of transcription factors playing fundamental roles during organogenesis. We have recently demonstrated the expression of Pax-2 during Xenopus embryogenesis [Heller, N, Brändli, AW (1997) Mech Dev 69, 83-104]. Here we report now the cloning and characterization of Xenopus Pax-5 and Pax-8, two orthologues of the Pax-2/5/8 gene family. Molecular phylogenetic analysis indicates that the amphibian Pax-2/5/8 genes are close relatives of their mammalian counterparts and that all vertebrate Pax-2/5/8 genes are derived from a single ancestral gene. Xenopus Pax-2/5/8 genes are expressed in spatially and temporally overlapping patterns during development of at least seven distinct tissues. Most strikingly, Xenopus Pax-8 was identified as the earliest marker of the prospective otic placode and of the intermediate mesoderm, indicating that Pax-8 may play a central role in auditory and excretory system development. Comparison of the expression patterns of fish, amphibian and mammalian Pax-2/5/8 genes revealed that the tissue-specificity of Pax-2/5/8 gene family expression is overall evolutionary conserved. The expression domains of individual orthologues can however vary in a species-specific manner. For example, the thyroid glands of mammals express Pax-8, while in Xenopus Pax-2 is expressed instead. Our findings indicate that differential silencing of Pax-2/5/8 gene expression may have occurred after the different classes of vertebrates began to evolve separately.
5.2.2. Introduction

The genesis of organs during embryonic development is regulated by inductive interactions and a complex process of activation and deactivation of genes controlling cellular fate and differentiation. Many of these developmental control genes code for transcription factors and are able to initiate expression of a cascade of subordinate genes necessary for proper organ development. It is therefore not surprising to find that mutations of these genes underlie a number of congenital syndromes (Engelkamp and van Heyningen, 1996). Pax genes constitute an important family of transcription factors playing a pivotal role as regulators of embryonic development (Dahl et al., 1997; Mansouri et al., 1996). The defining characteristics of this family is the presence of a 128-amino acid long paired domain encoding a unique DNA-binding motif (Chalepakis et al., 1991; Treisman et al., 1991). In addition, many of these genes contain a complete or truncated DNA-binding homeodomain and/or an octapeptide motif. The paired box was first identified in Drosophila, and homologs have been found in a variety of vertebrates and invertebrates. Nine Pax genes have been identified in human and mouse. Sequence similarity and gene structure indicate that the mammalian Pax gene family consists of four well-defined groups: group I (Pax-1/9), II (Pax-2/5/8), III (Pax-3/7), and IV (Pax-4/6) (Balczarek et al., 1997; Walther et al., 1991). During vertebrate development, most Pax genes are expressed in complex patterns within the central nervous system (Chalepakis et al., 1993; Mansouri et al., 1996).

Members of the Pax-2/5/8 gene family are also prominently expressed outside of the central nervous system implicating functions during development of the kidney (Pax-2 and Pax-8), the visceral arches (Pax-2), the thyroid gland (Pax-8), and B lymphocytes (Pax-5) (Adams et al., 1992; Asano and Gruss, 1992; Dressler et al., 1990; Heller and Brändli, 1997; Plachov et al., 1990). Pax-2/5/8 gene family members have recently been associated with human disease syndromes and specific developmental defects in mouse mutants. Patients with renal coloboma syndrome are heterozygous for PAX-2 mutations and have optic nerve colobomas, renal hypoplasia, and occasional hearing defects (Sanyanusin et al., 1995b). Natural and targeted mouse mutants demonstrate that Pax-2 is involved in multiple steps during urogenital development as demonstrated by the absence of kidneys, ureters and genital tracts (Favor et al., 1996; Torres et al., 1995). Similar to humans, mutant animals exhibit also defects in the eye (bilateral coloboma) and the inner ear (lack of cochlear outgrowth) (Favor et al., 1996; Torres et al., 1996). Expression of Pax-2 and Pax-5 overlaps spatially and temporally at the midbrain-hindbrain boundary.
(MHB) suggesting redundant functions. Indeed, homozygous mice deficient in either Pax-2 or Pax-5 show no gross defects in derivatives of the MHB (Schwarz et al., 1997; Urbánek et al., 1994). Analysis of double Pax-2/Pax-5 mutants reveals however a deletion of the cerebellum and alterations of the tectum and tegmentum (Schwarz et al., 1997). Early B-lymphopoiesis is critically dependent on Pax-5 gene function. In the fetal liver, Pax-5 is required for differentiation of the earliest B-lineage committed precursor cells (Nutt et al., 1997; Urbánek et al., 1994). Deregulation of PAX-5 in humans, elicited by a chromosomal translocation juxtaposing an IgH enhancer upstream of the PAX-5 locus, may contribute to the pathogenesis of a subset of non-Hodgkin’s lymphomas (Busslinger et al., 1996). Probably due to partially redundant functions provided by Pax-2 and Pax-5, Pax-8 mutant mice do not show obvious defects of the spinal cord, the MHB and the kidneys. Pax-8 is however required in the mouse for the formation of the follicular cells in the thyroid gland (Mansouri et al., 1998), and in humans heterozygosity for PAX-8 is associated with congenital hypothyroidism (Macchia et al., 1998). Together, these observations underscore the important role of the Pax-2/5/8 gene family in the control of various organogenesis processes.

Malformations of the kidneys occur sporadically, but on occasion they can also be familial. Affected individuals may have unilateral or bilateral disease which may take the form of either renal hypoplasia and/or agenesis. Renal malformations are believed to occur in 1:3,000 to 1:10,000 newborns and are the most commonest causes of chronic renal failure in the first years of life (Woolf, 1995). Early diagnosis and therapy therefore is absolutely critical. Recent evidence suggests that mutations of developmental control genes, such as PAX-2, SALL-1 and EYA-1, may cause familial renal malformations (Abdelhak et al., 1997; Kohlhase et al., 1998; Sanyanusin et al., 1995b). In order to understand the molecular basis of malformations of the kidney, it is therefore essential to decipher the other members in the cascades of molecular events underlying kidney organogenesis.

Early steps of kidney organogenesis, in particular the processes leading to the commitment of cells to the nephric fate and subsequent formation of the pronephric kidney, are difficult to study in the mammalian embryo, but can be more readily assessed in the amphibian embryo (Vize et al., 1997). We have recently reported the cloning of Xenopus Pax-2, and showed that expression of this gene is associated with the onset of pronephric kidney morphogenesis (Heller and Brändli, 1997). Here we describe now the cloning, molecular phylogeny and developmental expression of the Xenopus Pax-5 and Pax-8
genes. We found that expression of Pax-8 is associated with the prospective otic placode and the intermediate mesoderm. Expression is initiated at the same time point when otic fate and pronephric tubules become specified in the early neurula embryo. To the best of our knowledge, Pax-8 is the earliest gene known to be expressed in these tissues and may therefore be on top of molecular cascades controlling inner ear development and early kidney organogenesis.
5.2.3. Results

Cloning of Xenopus Pax-5 and Pax-8 cDNAs

A polymerase chain reaction (PCR) fragment encoding a peptide closely resembling mammalian Pax-5 had previously been isolated (Heller and Brändli, 1997). This fragment was used for screening of a Xenopus cDNA library, and a partial cDNA encoding the N-terminal 180 amino acids of a paired box protein was isolated. In mammals, the Pax-5 gene is transcribed from two distinct promotors, resulting in alternative splicing of different 5' end exons, 1A or 1B, to the common coding sequences of exons 2-10. Transcripts containing exon 1A are expressed predominantly in B-lymphocytes, while exon 1B containing transcripts are found in all Pax-5 expression domains (Busslinger et al., 1996). Sequence comparisons showed that the N-terminus of the Xenopus protein shared 13 out 14 amino acids with exon 1B of mouse and human Pax-5 (Fig. 13).

The missing 3' Xenopus Pax-5 sequences were isolated by rapid amplification of cDNA ends (RACE)-PCR. Subsequently, reverse transcription (RT)-PCR was performed to obtain cDNAs encoding the complete open reading frame (ORF) of Xenopus Pax-5. Sequence analysis led to the identification of five different classes of Xenopus Pax-5 cDNAs, Pax-5(1) to Pax-5(5). The observed cDNA variants are probably the result of alternative splicing events, as previously seen with mouse and human Pax-5 (Busslinger et al., 1996; Zwollo et al., 1997). Pax-5(1) encodes a protein that is collinear mammalian Pax-5 proteins and lacks the exon 9.1 found in zebrafish Pax-5 (Fig. 13). For Pax-5(2), usage of an alternative splice site located within intron 6 results in 108 additional nucleotides being retained between exon 6 and 7. The original reading frame remains however unaltered (Fig. 14A). Pax-5(3) encodes a truncated protein, which is generated by activation of an alternative splice site located in exon 2. This results in a deletion of 30 nucleotides and creates a premature stop codon at the junction site (Fig. 14B). Pax-5(4) and Pax-5(5) lack exon 2 or exon 7, respectively, resulting in truncated proteins (Fig. 14C, D). Nucleotide sequence comparisons suggest that all Pax-5 cDNAs are derived from transcripts of a single Xenopus Pax-5 gene (not shown).
Fig. 13. Sequence comparison of vertebrate Pax-5 proteins. *Xenopus* Pax-5(1) splice variant has been aligned with various vertebrate Pax-5 proteins. Human and mouse Pax-5 are shown with the alternatively spliced exon 1B (Busslinger et al., 1996). GenBank accession numbers to the human, mouse, and zebrafish Pax-5 sequences are listed in Fig. 16. The paired domain and the partial homeodomain have been highlighted by shading. The octapeptide domain has been underlined. Amino acids shared by all four proteins are boxed. Dashes indicate gaps that were introduced for optimal sequence alignment. Exons are numbered according to Pfeffer et al. (1998).
Results

Fig. 14. Splice variants of Xenopus Pax-5. (A) Xenopus Pax-5(2) is generated by use of an alternate 5' splice donor site present in intron 6. This leads to the introduction of 108 additional nucleotides. The 5' splice donor normally used is underlined. (B) Xenopus Pax-5(3) is generated by use of an alternate 5' splice donor site (underlined) present in exon 1. This leads to a deletion of 30 nucleotides (boxed) and to the creation of a new stop codon at the splice junction. (C, D) Xenopus Pax-5(4) and Pax-5(5) lack exon 2 or exon 7, respectively. In both cases, this leads to frame-shifts and to truncated proteins. Asterisks represent stop codons. Newly introduced amino acids are shown in bold.

In order to isolate Xenopus cDNAs encoding Pax-8, we designed degenerate PCR primers based on sequence motifs present in exons 6 and 8, which were conserved in Pax-8 but not in Pax-2 and Pax-5 proteins. An amplification product closely related to mammalian Pax-8 was isolated by RT-PCR and used for cDNA library screening. A single cDNA was isolated from a Xenopus adult kidney cDNA library. DNA sequencing revealed a partial ORF and 3' non-coding sequences. Sequence comparisons confirmed that the encoded partial protein sequence was collinear with the C-termini of vertebrate Pax-8 proteins and composed of exons 6 to 10 (Fig. 15).
### Results

| hPax-8 | C R L S I T D S Q S S S G P R X H G P R X H G P R X H G P R X H G P R X H |
| mPax-8 | C R L S I T D S Q S S S G P R X H G P R X H G P R X H G P R X H G P R X H |
| XPax-8 | C R L S I T D S Q S S S G P R X H G P R X H G P R X H G P R X H G P R X H |
| zPax-8 | C R L S I T D S Q S S S G P R X H G P R X H G P R X H G P R X H G P R X H |

**Fig. 15.** Comparison of vertebrate Pax-8 amino acid sequences. The deduced amino acid sequence of *Xenopus* Pax-8 was assembled from overlapping partial cDNA sequences. GenBank accession numbers to the human, mouse and zebrafish Pax-8 sequences are listed in Fig. 16. The partial homeodomain has been highlighted by shading. Amino acids shared by all four proteins are boxed. Dashes indicate gaps that were introduced for optimal sequence alignment. Exons are numbered according to Pfeffer et al. (1998).

The *Xenopus* Pax-8 sequence contains sequences homologous to the alternatively spliced exon 7.1 of human Pax-8a. This exon is absent in the Pax-8b splice variant (Kozmik et al., 1993). Sequence analysis of PCR-amplified Pax-8 cDNAs from *Xenopus* embryos revealed the existence of at least one further splice variant. This isoform was devoid of exon 7.1 leading to an in-frame fusion of exon 7 to exon 8 (not shown). Given that multiple mammalian Pax-8 isoforms have been described to date (Kozmik et al., 1993; Poleev et al., 1995), additional *Xenopus* Pax-8 splice variants may exist. Taken together, our
findings on the alternative splicing of *Xenopus* Pax-5 and Pax-8 transcripts confirm the evolutionary conservation of alternative splicing events for vertebrate Pax-2/5/8 genes as suggested previously (Heller and Brändli, 1997; Kozmik et al., 1993; Lun and Brand, 1998; Pfeffer et al., 1998).

**Conserved regions and phylogenetic relationships**

As demonstrated by sequence alignments, the *Xenopus* cDNAs encode proteins sharing a high degree of sequence identity to vertebrate Pax-5 and Pax-8, respectively (Fig. 13, 15). The *Xenopus* Pax-5 paired domain shows 98%, 97% and 93% amino acid identity to human Pax-5, Pax-2 and Pax-8, respectively. When compared to mouse and zebrafish Pax-8, the amino identities of the *Xenopus* Pax-5 paired domain was 98%. The characteristic octapeptide found in all Pax-2/5/8 genes was also present in *Xenopus* Pax-5 and was determined to be identical in sequence to the other vertebrate Pax-5 octapeptides (Fig. 13). Consistent with closest homology to Pax-5, the amino acid identities of the partial homeodomain of *Xenopus* Pax-5 were 93%, 63% and 43% to mammalian Pax-5, Pax-2 and Pax-8 proteins, respectively. The *Xenopus* Pax-5 homeodomain lacks the extensive deletion seen in zebrafish and fugu Pax-5 (Fig. 13). As this deletion is not found in amphibia and mammals, we suggest that the deletion event occurred during teleost fish evolution after separation from the tetrapod lineage. Comparison of the *Xenopus* Pax-8 homeodomain showed 57% amino acid identity to mammalian Pax-8 proteins, and 30% each to mammalian Pax-2 and Pax-5. The sequence identity over the entire C-terminal domain was 61% to mammalian Pax-8 proteins, but only 46% to zebrafish Pax-8. A comprehensive comparison of the sequence distances between chordate Pax-2/5/8 gene family members is given in Table 3. In summary, the amino acid conservation within the structural domains characteristic for the Pax-2/5/8 gene family allows unequivocal assignment of the cloned cDNAs as *Xenopus* orthologues of Pax-5 and Pax-8, respectively.

If one takes the previously reported pseudoallelic *Xenopus* Pax-2a and Pax-2b genes (Heller and Brändli, 1997) in account, the *Xenopus* genome appears to contain at least four genes of the Pax-2/5/8 family. Recently, novel members of the Pax-2/5/8 gene family have also been found in zebrafish (Pfeffer et al., 1998). The cephalochordate amphioxus is the closest relative of vertebrates and contains a single gene homologous to the vertebrate Pax-2/5/8 genes (N. D. Holland, personal communication). Molecular phylogenetic analysis was used to assess the relationship between the vertebrate Pax-2/5/8 gene family members and the amphioxus Pax-258 (Fig. 16).
Amino acids sequences encoded by the common exons 6-10 were aligned to calculate the sequence distances. The numbers indicate the percent of identical amino acids. GenBank accession numbers can be found in the legend to Figure 4. a, amphioxus; h, human; m, mouse; X, Xenopus; z, zebrafish.

Since for some of the genes only partial sequences were available, the alignment included the common exons 6 to 10. The resulting phylogenetic tree supports the hypothesis of multiple genome or large-scale gene duplications during vertebrate evolution (Holland and Garcia-Fernandez, 1996; Sharman and Holland, 1996). We also infer from the tree that the vertebrate Pax-2/5/8 gene family has evolved from a common ancestral gene.

Several fish and amphibian species, including zebrafish and *Xenopus laevis*, appear to have undergone partial or complete genome duplications, since their lineages separated from the stem leading to higher vertebrates (Graf, 1996; Postlethwait et al., 1998). These findings are supported by the molecular phylogeny of the Pax-2/5/8 gene family which places the Pax-2 gene
Results

duplications after the separation of fish and amphibia from the lineage leading to the mammalian Pax-2 genes (Fig. 16). Interestingly, the zebrafish Pax-2 proteins share an amino acid identity of 93%, while the Xenopus Pax-2 proteins have an identity of more than 98%. It is therefore likely that the Xenopus Pax-2 duplication occurred more recently during vertebrate evolution than the zebrafish Pax-2 duplication.

Fig. 16. Phylogenetic analysis of the chordate Pax-2/5/8 gene family. The phylogenetic tree is based on multiple sequence alignments of amino acids encoded by the common exons 6-10. The scale bar measures the distance between the sequences. Units indicate the number of substitution events. The evolutionary distance between any two sequences is the sum of the horizontal branch length separating them. Vertical distances are for illustration purposes only. Abbreviations: a, amphioxus; h, human; m, mouse; X, Xenopus; and z, zebrafish. The GenBank accession numbers for the Pax sequences used are: aPax-2, AF053762; hPax-2, M89470; hPax-5, M96944; hPax-8, L19606; mPax-2, X55781; mPax-5, M97013; mPax-8, X57487; XPax-2a, Y10119; XPax-2b, Y10120; zPax-2.1, X63961; zPax-2.2, AF072547; zPax-5, AF072548; zPax-8, AF072549.

Embryonic expression of Xenopus Pax-5

The developmental expression of Xenopus Pax-5 was examined by whole-mount in situ hybridization. The expression was first detected faintly in mid-neurula embryos from stage 16 on in the folding neural tube. Transcripts were associated with two distinct patches of cells flanking the midline and located in the anterior third of the neural plate (not shown). With the completion of neurulation, the Pax-5 expressing cells became localized to the MHB (Fig. 17A). In a cross section, Pax-5 transcripts were highly expressed in the dorsal region of the MHB (Fig. 17D).
Fig. 17. Expression of Pax-5 in during *Xenopus* embryogenesis and of Pax-2 in the thyroid anlage. Transcripts for Pax-5 (A-E) and Pax-2 (F, G) were detected by whole-mount *in situ* hybridization. Sections of embryos stained in whole mount were cut at 50 μm. A-C: Pax-5 expression can be detected at the MHB (arrowhead) and faintly in the otic vesicle (arrow). Lateral views of stage 24 (A), 30 (B), and 38 (C) embryos are shown. Horizontal lines in (A) indicate the levels of the sections in (D) and (E). D, E: Horizontal sections through a stage 24 embryo to illustrate Pax-5 expression at the MHB (D) and in the otic vesicle (E). F, G: Pax-2 expression in the head region of a stage 34 embryo. Arrowheads indicate the stained thyroid anlage. Lateral (A-C, F) and ventral (G) views with anterior to the left are shown.

Robust expression of Pax-5 at the MHB continued through stage 30 and 38 (Fig. 17B, C) without decreasing in stage 40 embryos, the latest stage examined (not shown). Unlike the situation reported for mouse Pax-5 (Rowitch and McMahon, 1995), the expression of *Xenopus* Pax-5 was absent from regions adjacent to MHB, notably the posterior midbrain and anterior hindbrain. *Xenopus* Pax-5 expression could also not be detected in the other parts of the hindbrain and developing spinal cord. Inspection of tailbud-stage embryos revealed, however, faint Pax-5 expression in the region of the developing otic vesicle (Fig. 17A). A cross sections of a stage 24 embryo showed that Pax-5
Results

transcripts were localized to the ear placode (Fig. 17E). Expression was transient and restricted to the period of otic vesicle invagination from stage 21 to stage 27. As Pax-5 is essential for B-lymphopoiesis, we investigated whether Pax-5 could be detected in embryonic regions implicated in haematopoiesis. Primitive haematopoiesis in the ventral blood islands produces predominantly erythrocytes, while definitive blood cells, representing all blood cell lineages arise predominantly from the dorsal lateral plate region surrounding the pronephric duct (Huber and Zon, 1998). Stem cells of the B-lymphocyte lineage colonize the liver starting from stage 39, where they differentiate to become pre-B cells at stage 46 (Hadji-Azimi et al., 1982). Careful inspection of embryos as old as stage 40 failed to reveal Pax-5 expressing cells in any of the implicated regions. This finding indicates that the onset of B cell differentiation must occur after stage 40 in *Xenopus*.

Embryonic expression of *Xenopus* Pax-8

Pax-8 expression is first detected by *in situ* hybridization at stage 12/13 in the early neurula embryo (Fig. 18A). Two patches of cells are detected in the lateral regions of the embryo. The anterior expression domain marks the prospective otic region giving later rise to the otic vesicle. By late neurula stages, Pax-8 expression in the anterior domain becomes gradually concentrated in the otic placode (Fig. 18B, C). Pax-8 transcripts remain associated with the developing ear throughout the invagination phase. Later, expression is down-regulated after stage 34. The posterior expression domain of the neurula stage embryo is restricted to the intermediate mesoderm (Fig. 6A). Later, Pax-8 transcripts mark the pronephric primordium (Fig. 18B). After neurulation, expression can be seen in both the pronephric tubule and duct anlage (Fig. 6C). During pronephric kidney morphogenesis, Pax-8 expression is found in the pronephric tubules and the elongating duct (Fig. 18D). By stage 31, expression in the duct gradually ceases, while transcription in the differentiating pronephric tubules remains at high levels at least until stage 36/37, when the pronephric kidney becomes functional (Fig. 18E,F). Our findings identify Pax-8 as the earliest molecular marker for cells fated to become primordia of the otic system and the pronephric kidney, respectively. Analysis of tadpole stage embryos revealed the hindbrain and spinal cord as a third expression domain from stage 34 on (Fig. 18F). Though Pax-8 is required for the formation of follicular cells in the thyroid gland of higher vertebrates (Macchia et al., 1998; Mansouri et al., 1998), we failed to detect *Xenopus* Pax-8 transcripts in the region of the thyroid anlage.
Fig. 18. Expression of Pax-8 during *Xenopus* embryogenesis. Transcripts for Pax-8 were detected by whole-mount *in situ* hybridization. Lateral views are shown with anterior to the left. (A) Stage 12/13 embryo. Pax-8 transcripts are detected in the prospective otic region (arrow) and faintly in the intermediate mesoderm (arrowhead). (B) Stage 17 embryo. Up-regulation of Pax-8 in the pronephric anlage is evident (arrowhead). (C) Stage 22 embryo. Pax-8 expression in the pronephric tubule (arrow) and pronephric duct anlage (arrowhead) are indicated. (D) Stage 25 embryo. Pax-8 expression in the otic vesicle has started to cease. (E) Stage 31 embryo. Pax-8 expression in the pronephric duct (arrowhead) gradually decreases. (F) Stage 34 embryo. Pax-8 transcripts are detected in the hindbrain (arrow) and spinal cord. Strong expression remains with pronephric tubules (arrowhead).

**Expression of Xenopus Pax-2 in the thyroid gland**

The absence of *Xenopus* Pax-8 expression in the thyroid gland prompted us therefore to investigate whether other Pax-2/5/8 family member might be expressed instead. In *Xenopus*, the thyroid anlage appears at stage 33/34 as a posteriorly directed finger-shaped protrusion of the oro-pharyngeal floor at the level of the first visceral pouch (Nieuwkoop and Faber, 1994). Analysis of stage
Results

34 embryos stained for Pax-5 were negative (not shown), while embryos stained for Pax-2 revealed an elongated patch of cells located at the ventral midline (Fig. 17F,G). Location and pattern of staining identifies this tissue as the thyroid anlage. From stage 39, when the thyroid gland splits into two lobes, Pax-2 expression gradually starts to decrease (not shown). Therefore, *Xenopus* embryos express Pax-2 in place of Pax-8 and expression occurs well before follicular cells can be identified by morphology at stage 49/50.

*Induction of Pax-8 expression in explant cultures*

Animal cap assays were used to investigate the induction of Pax-5 and Pax-8 expression in primitive ectoderm. Animal caps were cultured in the presence or absence of growth factors and/or retinoic acid and Pax gene expression was assayed by whole mount *in situ* hybridization. None of the culture conditions tested resulted in induction of Pax-5 expression (not shown). Likewise, Pax-8 transcripts were not expressed in control cultures or in those treated with retinoic acid (100 μM) or bFGF (100 ng/ml) (Fig. 19A, B; and not shown). Analysis of cultures containing activin (5 ng/ml) alone revealed patches of Pax-8 expressing cells in a third of the animal caps (n=22; Fig. 19C). Activin in combination with retinoic acid however was most effective by inducing Pax-8 expression in over 90% of the explants (n=25). Typically, strong Pax-8 expression was found in patches of tissue located in the vicinity of the characteristic oedema seen frequently in these cultures (Fig. 19D). The differential induction of Pax-8 expression in explant cultures could be confirmed by RT-PCR (not shown). Interestingly, the culture conditions permissive to strong expression of Pax-8 were previously shown to promote the induction of pronephric tubules at very high frequency (Moriya et al., 1993; Uochi and Asashima, 1996). It is therefore likely that Pax-8 expression observed in our cultures is associated with pronephric tissues.
Fig. 19. Induction of Pax-8 expression in explant cultures. Animal caps were cultured either untreated (A), or with retinoic acid (B), activin (C) or a combination of activin and retinoic acid (D). Expression of Pax-8 was assayed by whole mount in situ hybridization once reference embryos reached stage 28. Arrowheads indicate patches of tissues positive for Pax-8 expression.
5.2.4. Discussion

Evolution of the Pax-2/5/8 gene family

Members of the Pax-2/5/8 gene family were first identified in higher vertebrates (Adams et al., 1992; Asano and Gruss, 1992; Dressler et al., 1990; Plachov et al., 1990), but the recent identification of invertebrate Pax-2/5/8 gene family members in hydra (Sun et al., 1997), C. elegans (Chamberlin et al., 1997; Czerny et al., 1997), Drosophila (Czerny et al., 1997; Fu and Noll, 1997), sea urchins (Czerny et al., 1997), ascidians (Wada et al., 1998), and amphioxus (N.D. Holland, personal communication) has revealed that this gene family is of ancient evolutionary origin. Pax genes appear to have evolved solely in the animal kingdom, since to date no Pax homologs have been reported from plants, fungi, yeasts, or other distant relatives to animals. It has been proposed that Pax genes evolved from a single gene present in the ancestors of all triploblastic animals (protostomes and deuterostomes) and that the encoded protein contained a paired domain, a homeodomain, and an octapeptide (Balczarek et al., 1997; Noll, 1993). The four major groups (Pax-1/9; Pax-2/5/8, Pax-3/7; Pax-4/6) arose by gene duplications prior to the Cambrian divergence of the major triploblastic animal lineages. After the separation of protostomes and deuterostomes, genome and/or gene duplications increased the number of Pax genes in each Pax group. Consistent with this scenario, mammalian genomes contain three genes of the Pax-2/5/8 family.

It has been unclear when during chordate evolution the duplications producing the three mammalian Pax-2/5/8 genes took place. Based on phylogenetic analysis of the mammalian Pax genes, it had been originally proposed that the Pax-2/5/8 duplications occurred after the separation of the lines leading to fish and mammals (Noll, 1993). This question has now been conclusively resolved with the cloning of Pax-2/5/8 gene family members from ascidians (Wada et al., 1998), amphioxus (N. D. Holland, personal communication), zebrafish (Pfeffer et al., 1998), and Xenopus (Heller and Brändli, 1997; present study). The genomes of ascidians and amphioxus were found to contain a single ancestral Pax-2/5/8 gene, while zebrafish and amphibian genomes harbor orthologues to each of the three mammalian Pax-2/5/8 genes. Therefore, the duplication events occurred after the separation of the cephalochordate lineage, but prior to the divergence of teleost fish, from the stem leading to the tetrapods. This finding is supported by the molecular phylogeny of the Pax-2/5/8 gene family matching the accepted evolutionary relationship of species in the chordate phylum (Fig. 16). The phylogenetic tree
also strongly supports the previously suggested scenario implying that a first gene duplication generated Pax-8 and the precursor gene of Pax-2 and Pax-5. Subsequently, a second duplication gave rise to Pax-2 and Pax-5 (Balczarek et al., 1997; Noll, 1993). A more comprehensive understanding of Pax-2/5/8 gene duplication during early vertebrate evolution will have to await the cloning of Pax-2/5/8 gene family members from jawless vertebrates (hagfish, lamprey).

Pax genes in the developing inner ear

A comparative analysis of the expression profiles of the Xenopus Pax-2/5/8 family members is shown in Fig. 20. We find that Xenopus Pax-2/5/8 genes are expressed in a spatially and temporally overlapping manner during the development of seven embryonic structures. Interestingly, tissue-specific expression of Pax-2/5/8 genes occurs very early in embryogenesis, usually well before these structures become morphologically discernible. This is consistent with proposed roles for Pax-2/5/8 genes in defining, controlling or participating in the cascade of events leading to the proper formation of many tissues and organs of the embryo. For example, development of the vertebrate inner ear has been shown to be critically dependent on Pax-2 function (Favor et al., 1996; Torres et al., 1996). The first morphologically discernible structures of inner ear development are the otic placodes arising from ectodermal thickenings lateral to the prospective hindbrain. The placodes subsequently invaginate to form the otic cup and eventually the closed otic vesicle. The otic vesicle undergoes a distinct period of proliferation and complex morphogenetic movement prior to differentiation of specific cell types (Fekete, 1996; Torres and Giráldez, 1998).

In the mouse, Pax-2 expression occurs from the otic cup to the otic vesicle stage (Nornes et al., 1990), and mutations of Pax-2 result in agenesis of the cochlea, the auditory apparatus of the ear (Favor et al., 1996; Torres et al., 1996). The cloning and characterization of Pax-2/5/8 genes from lower vertebrates provides now evidence for possible roles of other family members in ear development. Pax-5 expression was found to occur during invagination of the otic placode in Xenopus (Fig. 17) and zebrafish (Pfeffer et al., 1998). In the mouse, however, the Pax-5 gene is no longer expressed in the otic region indicating that its activity is dispensable for mammalian ear development. This view is further supported by the absence of auditory deficiencies in Pax-5 mutant animals (Urbánek et al., 1994). Surprisingly, analysis of Pax-8 revealed a significantly earlier onset of gene expression in the region of the prospective otic vesicle in comparison to Pax-2 and Pax-5 (Fig. 18, 20). The acquisition of
Results

placodal competence by a stripe of ectoderm surrounding the anterior neural plate is considered the earliest step in ear development and starts during gastrulation. Subsequently, the competence of the placodal area in the region of the future otic field becomes restricted and finally commitment to the otic fate occurs (Torres and Giráldez, 1998).

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Pax-2</th>
<th>Pax-5</th>
<th>Pax-8</th>
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<tr>
<td>12</td>
<td></td>
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<tr>
<td>14</td>
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<td>16</td>
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<td>18</td>
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<td>20</td>
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<td>22</td>
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<td>24</td>
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<tr>
<td>26</td>
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<td>n.d.</td>
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<tr>
<td>28</td>
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<tr>
<td>30</td>
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<tr>
<td>32</td>
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<td>n.d.</td>
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<tr>
<td>34</td>
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<td>n.d.</td>
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<tr>
<td>36</td>
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<td>n.d.</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td>40</td>
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<td>n.d.</td>
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</table>

Fig. 20. Temporal regulation of gene expression for members of the Pax-2/5/8 gene family during *Xenopus* embryogenesis. The duration of gene expression is schematically shown based on results from whole-mount *in situ* hybridization studies. Pax-2a and Pax-2b expression cannot be resolved into separate patterns (Heller and Brändli, 1997). The numbers shown indicate developmental stages according to Nieuwkoop and Faber (1994). n.d., no expression detected.
Results

Explant experiments in *Xenopus* suggest that the otic placodes become committed during early neurula stages (Gallagher et al., 1996). Very little is however understood at the molecular level. Several genes (Dlx-3, Msx-1, Nkx-5.1, Six-1) are expressed in sensory placodes, but none is specific for a single placode (Torres and Giráldez, 1998), nor are they expressed as early as Pax-8. To the best of our knowledge, Pax-8 constitutes the first molecular marker for the prospective otic placodal region, and its temporal expression coincides with the period of otic fate determination. It remains however to be established whether Pax-8 fulfills any critical function during the initial steps of inner ear development. Pax-8 expression in the developing ear is evolutionary conserved between zebrafish, *Xenopus* and mouse (Pfeffer et al., 1998; present study). It will be therefore of interest to determine whether the recently reported Pax-8 deficient mice (Mansouri et al., 1998) exhibit any auditory system defects.

**Pax genes in kidney organogenesis**

*Xenopus* Pax-8 expression begins at stage 12/13 in the intermediate mesoderm of the early neurula embryo, condenses to form the pronephric region, and continues throughout morphogenesis (Fig. 18). The first morphological indication of the pronephric anlage is observed at stage 21 (Nieuwkoop and Faber, 1994). Initiation of Pax-8 expression therefore precedes the onset of morphological differentiation of the pronephric kidney by several hours. Xlim-1 is a second marker expressed in the pronephric lineage prior to morphogenesis (Taira et al, 1992; N. Heller and A.W. Brändli, unpublished observation). But unlike Pax-8, early mesodermal Xlim-1 expression is not specific to the intermediate mesoderm only, but also extends into the adjacent lateral plate mesoderm (Taira et al., 1992; data not shown). Besides Pax-8, Pax-2 is the only other Pax-2/5/8 family member with expression in the developing kidney (Fig. 20, Table 4). In *Xenopus*, initiation of Pax-2 expression occurs with the onset of pronephric kidney morphogenesis at stage 21 (Heller and Brändli, 1997). This places Pax-2 clearly downstream of Pax-8. Similarly in zebrafish, Pax-8 precedes expression of the Pax-2 orthologue Pax-2.1 in the pronephric kidney [Pfeffer et al., 1998] indicating evolutionary conservation of the sequence of Pax gene expression in the developing excretory systems of lower vertebrates. It remains to be established whether this applies also to mammals. Taken together, Pax-8 can be considered the earliest marker of the intermediate mesoderm and thus of the pronephric lineage. This finding is surprising, since on the basis of comparative expression studies in the developing mouse metanephric kidney (Plachov et al., 1990), Pax-8 has been
placed downstream of Pax-2 in the molecular cascade controlling nephrogenesis.

What role might Pax-8 play during early kidney organogenesis? Specification of pronephric tubules and ducts occurs in *Xenopus* between stage 12.5 and 14 (Brennan et al., 1998). This correlates precisely with the onset of Pax-8 expression. Further, animal cap explants cultured under conditions permissive to pronephric tubule induction express high levels of Pax-8 (Fig. 19). Pax-8 may therefore be an early response gene to pronephric inducers and function in specifying the pronephric fate. The phenotypes of Pax-2 deficient animals are also consistent with this proposed early function of Pax-8 in kidney organogenesis. Zebrafish and mouse mutants form rudimentary pronephric ducts which fail to elongate and subsequently degenerate (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995). The transient expression of Pax-8 in the elongating pronephric duct (Fig. 18D-F) may be sufficient to permit initiation of pronephric duct formation even in the absence of Pax-2. Surprisingly, mice deficient in Pax-8 do apparently not exhibit kidney defects (Mansouri et al., 1998) indicating that Pax-2 can fully compensate for the absence of Pax-8 in mutant animals. It will therefore be important to analyze mutants deficient in both Pax-2 and Pax-8 to establish whether Pax gene function is required at the earliest stages of kidney organogenesis, for example, to specify cells of the intermediate mesoderm to the pronephric fate as suggested here.

*Evolutionary conservation and species-specific differences of Pax-2/5/8 gene family expression*

A comparison of the expression profiles of zebrafish, *Xenopus* and mouse defines eight structures of the developing vertebrate embryo associated with Pax-2/5/8 expression: the MHB, the optic system, the inner ear, the hindbrain/spinal cord, the excretory system, the thyroid gland, the visceral arches and the B-lymphocyte lineage. Table 4 compiles the data for the six tissues where sufficient information covering all three species is currently available.

Overall the expression patterns of the Pax-2/5/8 genes are well conserved among the distantly related vertebrates. Each species was found to express at least one parologue in each of the six tissues investigated. Expression of Pax-2/5/8 genes in these tissues can therefore be considered evolutionary ancient. In support of this view, expression of HrPax-258, an ascidian homologue of the Pax-2/5/8 gene family, is detected in a region of the neural tube considered to be homologous to the MHB region, in the atrial primordia, a structure possibly
homologous to the vertebrate ear, and in the primordial pharynx (Wada et al., 1998). It therefore appears that the neural tube, sensory placodes (e.g., inner ear) and pharyngeal tissues (e.g., thyroid gland) represent the evolutionary oldest structures associated with Pax-2/5/8 gene function in the chordate phylum.

Table 4. Comparison of the embryonic expression domains of vertebrate Pax-2/5/8 gene family members

<table>
<thead>
<tr>
<th></th>
<th>mhb</th>
<th>eye</th>
<th>ear</th>
<th>hb/sc</th>
<th>kidney</th>
<th>thyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPax-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>XPax-2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>zPax-2.1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>zPax-2.2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>?</td>
</tr>
<tr>
<td>mPax-5</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>zPax-5</td>
<td>+</td>
<td>n.d.</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>?</td>
</tr>
<tr>
<td>mPax-8</td>
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<td>n.d.</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>XPax-8</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>n.d.</td>
</tr>
<tr>
<td>zPax-8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>?</td>
</tr>
</tbody>
</table>

m, mouse; X, Xenopus; z, zebrafish; mhb, midbrain-hindbrain boundary; hb/sc, hindbrain/spinal cord; n.d., no expression detected; +, expression detected; ?, no data available.

Expression of Pax-2/5/8 genes in other tissues, particularly in the excretory organs may therefore be regarded as a newly acquired feature of more advanced chordates. Interestingly, the cephalochordates have a subdivided neural tube and structures homologous to the thyroid gland, the visceral arches, several sensory organs as well as clearly defined excretory organs (Whittaker, 1997), and all these tissues were found to express the amphioxus Pax-258 gene (N. D. Holland, personal communication). This suggests that the tissue-specificity of Pax-2/5/8 gene family expression was established before the proposed duplication events took place resulting in the present day complexity of the vertebrate Pax-2/5/8 gene family. This scenario also provides an explanation why several tissues of the vertebrate embryo express more than one Pax-2/5/8 paralogue despite apparently redundant
functions. Furthermore, it implies that each duplicated Pax-2/5/8 gene must have had originally the potential to be expressed in all the possible tissues. Indeed, we find that *Xenopus* Pax-2 is expressed in all six tissues (Table 4). This suggests that *Xenopus* Pax-2 may have retained gene regulatory sequences most closely resembling those of the ancestral Pax-258 gene.

While many aspects of the expression patterns of Pax-2/5/8 genes are evolutionarily conserved, some species-specific differences are nevertheless observed. For example, the mammalian thyroid gland expresses Pax-8, while *Xenopus* embryos express Pax-2 instead. This observation has several implications. First, it provides direct evidence that Pax-2 and Pax-8 can functionally substitute each other *in vivo*. Secondly, while the Pax-2/5/8 coding regions are highly conserved, both structurally and functionally, between vertebrate species, the enhancer elements appear to have diverged. This finding is consistent with the observation that the coding regions of master control genes are conservative in their evolution, whereas the enhancer elements undergo rapid modification following gene duplication (Sidow, 1996). Finally, differential silencing of Pax-2/5/8 expression must have occurred for some of the embryonic tissues after the separation of amphibian and mammalian lineages during vertebrate evolution. Comparative studies of the gene regulatory sequences of vertebrate Pax-2/5/8 orthologues may therefore provide important insights on the evolution of tissue-specific enhancer elements.
5.2.5. Materials and Methods

Embryo collection and animal cap assays

*Xenopus* embryos were obtained from *in vitro* fertilizations as described previously (Brändli and Kirschner, 1995). Dissection of animal caps and culture conditions are described in (Heller and Brändli, 1997). Retinoic acid was dissolved in ethanol and used at a final concentration of 100 μM (Uochi and Asashima, 1996).

Cloning of Xenopus Pax-5 cDNAs

Partial cDNAs encoding *Xenopus* Pax-5 were isolated by screening a λZAPII *Xenopus* stage 28 head cDNA library (Hemmati-Brivanlou et al., 1991). A PCR-generated Pax-5 cDNA which had been previously isolated (Heller and Brändli, 1997) was used to generate [32P]-labeled probes by random-priming. Library screening was carried out under conditions of high stringency using standard methods. A single positive clone was identified, purified and converted into a Bluescript plasmid using the Rapid Excision Kit (Stratagene). The 2-kb plasmid pH.D.PA14 was sequenced on both strands by the double-strand dideoxy-chain termination method using T7 DNA polymerase (Sequenase Version 2.0; Amersham Life Science). A cDNA encoding the C-terminus of *Xenopus* Pax-5 was isolated by 3'RACE using the 5'3' RACE kit (Boehringer Mannheim) according to the manufacturer’s recommendations. Finally, cDNAs encoding the entire ORF of Pax-5 splice variants were amplified by RT-PCR using primers corresponding to nucleotides located in the 5' non-coding region flanking the start codon and at the 3' end of the ORF including the termination codon, respectively.

Cloning of Xenopus Pax-8 cDNAs

Degenerate PCR primers corresponding to regions conserved between zebrafish, mouse and human Pax-8 were designed and used for RT-PCR amplification. The following degenerate primer pair was used: XPA-7, coding for the target peptides DDSDQ(D/E); XPA-4, coding for YPPHIP. RNA isolation and RT-PCR were carried out as described (Heller and Brändli, 1997). The amplification product was purified using the GeneClean Spin Kit (Bio 101), subcloned into Bluescript II SK + (Stratagene) and sequenced. The PCR-generated probe containing the *Xenopus* Pax-8 sequence was used to screen
an adult *Xenopus* kidney cDNA library (Stratagene) at high stringency. A single positive clone was identified, purified, and converted to a Bluescript plasmid. This plasmid BS.KLPax8.1 contained a partial cDNA of 3 kb, and was sequenced as described above. The sequences for *Xenopus* Pax-5 and Pax-8 have been deposited in the GenBank/EMBL database (accession nos. AJ010503 and AJ010504).

**Sequence analysis**

Homology searches were performed using BLAST programs (Altschul et al., 1990). Amino acid sequences were aligned with the MegAlign program (DNASTar) using the Clustal method with either a PAM250 residue weight or an identity table. The aligned amino acid sequences were used to construct phylogenetic trees with the Neighbor-Joining algorithm (Saitou and Nei, 1987).

**Whole mount in situ hybridization analysis**

Whole mount *in situ* hybridizations, vibratome sectioning and photography were performed as previously described (Heller and Brändli, 1997). Single-stranded RNA molecules were labeled with digoxigenin-UTP (Boehringer Mannheim), after hybridization the labeled RNA probes were detected with anti-digoxigenin antibody coupled to alkaline phosphatase. Plasmids BS.14A6 and BS.KLPax8.1 were used for synthesis of antisense probes directed against Pax-5 and Pax-8 transcripts, respectively. Plasmid BS.14A6 was generated by subcloning the partial ORF of Pax-5 from HD.PA14 into Bluescript II SK (+). Plasmids for Pax-2 antisense probe synthesis can be found elsewhere (Heller and Brändli, 1997).
Acknowledgements

We thank Meinrad Busslinger and Nick Holland for sharing data on zebrafish Pax-8 and amphioxus Pax-258 prior to publication. We also thank Nick Holland for his insightful comments on the manuscript. A.W.B. is a recipient of a Career Development Award of the Swiss National Science Foundation (START-Grant 31-38807.93). This work was supported by Grant 31-50755.97 from the Swiss National Science Foundation to A.W.B.
5.3. Overexpression experiments reveal distinct roles for Pax-2 splice forms in neural development and pronephric kidney organogenesis

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Manuscript in preparation
5.3.1. Abstract

Pax genes are a family of transcription factors playing fundamental roles during embryonic development. We have recently reported the isolation of cDNAs encoding nine *Xenopus* Pax-2 isoforms [Heller and Brändli (1997) Mech. Dev. 69, 83-104]. The Pax-2 isoforms retain DNA-binding domains, but can differ significantly in their C-termini and thus probably have different transactivation properties. Pax-2 expression is confined to the nervous system, sensory organs, the visceral arches, and the developing excretory system. To elucidate Pax-2 gene functions during *Xenopus* embryogenesis, RNA levels of the individual Pax-2 isoforms were artificially altered by injection of synthetic Pax-2 RNA. The effects of ectopic Pax-2 expression were monitored by *in situ* hybridization using antisense probes directed against tissue-specific marker genes. Overexpression of Pax-2 led to an expansion of the expression domain of specific midbrain-hindbrain boundary (MHB) markers such as En-2 and Pax-5. Furthermore, ectopic cells positive for these MHB marker genes were detected in the vicinity to the MHB. In the developing kidney, ectopic Pax-2 expression resulted in a massive enlargement of pronephric tissue as monitored by marker gene expression and sectioning. Pax-2 isoforms with a minimal transactivation domain were sufficient to elicit the pronephric phenotype, while Pax-2 proteins with additional exons were necessary to induce effects at the MHB. Our results suggest that Pax-2 isoforms may function differentially in the developing nervous system and pronephric kidney. Since we have previously shown that alternative splicing of Pax-2 transcripts does not occur in a tissue-specific manner, the observed phenotypes may be achieved by Pax-2 isoforms synergizing with tissue-specific cofactors.
5.3.2. Introduction

Over the last years it has become apparent that transcription factors play a major role in the development of an organism, directing or influencing fundamental processes like cell lineage specification, morphogenesis and cell differentiation (Kessel and Gruss, 1990; Rothenpieler, 1996). But to date, we are still far from having completely deciphered the basic biological mechanisms which lead to tissue patterning and organogenesis.

Pax proteins constitute a small class of transcription factors that share a highly conserved DNA-binding sequence, the paired domain (Chalepakis et al., 1991; Treisman et al., 1991). Most Pax proteins also contain other conserved motifs such as the paired-type homeodomain and the octapeptide. Pax proteins diverge mainly in their C-termini which contain sequence motifs mediating transcriptional activation (Chalepakis et al., 1994; Czerny and Busslinger, 1995; Fickenscher et al., 1993). In vertebrates, nine Pax genes have been identified (St-Onge et al., 1995). On the basis of sequence similarity and gene structure, the vertebrate Pax gene family is subdivided into four paralogous groups: group I (Pax-1/9), II (Pax-3/7), III (Pax-2/5/8), and IV (Pax-4/6) (Balczarek et al., 1997; Walther et al., 1991). Studies over the last ten years have identified Pax genes as important developmental control genes involved in the formation of various structures and organs in the embryo (Dahl et al., 1997; Mansouri et al., 1996).

The vertebrate excretory system develops from the intermediate mesoderm by successive formation of three distinct kidneys along the rostrocaudal axis of the embryo namely the pronephric, the mesonephric, and the metanephric kidney (Saxén, 1987). The pronephric kidney composed of the pronephros and the pronephric duct is established as the earliest kidney and represent the simplest form of an excretory organ in vertebrates. While the pronephros remains a transitory structure, the pronephric duct constitutes the central component of the vertebrate excretory system. It induces the morphogenesis and differentiation of the mesonephric and metanephric nephrogenic mesenchyme into tubular structures (Herzlinger, 1995).

Pax-2 and Pax-8 expression is associated with all three stages of vertebrate kidney organogenesis (Dressler et al., 1990; Dressler and Douglass, 1992; Heller and Brändli, 1997; Heller and Brändli, 1999; Krauss et al., 1991; Pfeffer et al., 1998; Plachov et al., 1990). In the pronephric kidney, Pax-8 was found to be the earliest marker of the intermediate mesoderm and thus of the pronephric lineage, whereas Pax-2 gene expression was associated with the onset of pronephric kidney morphogenesis. The chronological order of initiation of Pax-8 and Pax-2 expression indicates that Pax-8 might have a role in the
Results

specification of the pronephric tubules and ducts while Pax-2 might function in pronephric kidney morphogenesis and/or differentiation in Xenopus (Heller and Brändli, 1997; Heller and Brändli, 1999). A number of mutations in the Pax-2 gene establish a central role for Pax-2 in kidney organogenesis (Favor et al., 1996; Keller et al., 1994; Sanyanusin et al., 1995a; Sanyanusin et al., 1995b; Torres et al., 1995). During embryogenesis, Pax-2 deficient animals form rudimentary pronephric ducts that fail to elongate and subsequently degenerate at the timepoint when they should begin to interact with the nephrogenic mesenchyme (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995). Consequently, Pax-2 knockout mice lack mesonephric and metanephric kidneys, ureters and genital tracts. This had led to the proposal that Pax-2 might represent a primary regulator of kidney organogenesis (Torres et al., 1995). The precise role of Pax-2 in the process is however still poorly understood.

The midbrain-hindbrain boundary (MHB) has been identified as an important signaling center that controls the establishment of the midbrain and the cerebellum during development of the central nervous system (Joyner, 1996). Several transcription factors and secreted factors are required during development of the MHB such as En-1 and En-2 (Millen et al., 1994; Wurst et al., 1994), Otx-1 and Otx-2 (Acampora et al., 1997; Ang et al., 1996), Gbx-2 (Wassarman et al., 1997), Wnt-1 (McMahon and Bradley, 1990), and FGF-8 (Crossley et al., 1996; Meyers et al., 1998; Reifers et al., 1998). Expression of Pax-2, Pax-5 and Pax-8 overlaps spatially and temporally at the MHB suggesting redundant functions (Asano and Gruss, 1992; Krauss et al., 1991; Nornes et al., 1990; Pfeffer et al., 1998; Plachov et al., 1990). Indeed, homozygous mice deficient in either Pax-2, Pax-5 or Pax-8 show no or only mild defects in derivatives of the MHB (Mansouri et al., 1998; Schwarz et al., 1997; Urbánek et al., 1994). Analysis of double Pax-2/Pax-5 mutants reveals, however, a deletion of the cerebellum and alterations of the tectum and tegmentum (Schwarz et al., 1997). In Xenopus, we have isolated cDNAs for all three Pax-2/5/8 gene members (Heller and Brändli, 1997; Heller and Brändli, 1999). This allows us to analyze Pax gene function in gain-of-function experiments and to proof functional redundancy of these genes in vivo.

Alternative splicing is an important mechanism generating protein diversity from a single gene (Smith et al., 1989) and is a common phenomenon for Pax genes. Multiple splice products have been reported for nearly all members of the Pax gene family. Transcripts that either contain or lack particular exons may have entirely different biochemical properties at the protein level (Foulkes and
Sassone-Corsi, 1992). Indeed, in vitro studies have revealed that alternative splicing can modulate DNA-binding affinity and specificity as well as transactivation activity. For example, insertion of an additional exon in the paired domain of Pax-6 results in modification of the DNA binding specificity suggesting that the two isoforms regulate different sets of target genes (Epstein et al., 1994b). Furthermore, alternate use of consensus 3' splice sites lead to a deletion of three nucleotides in the paired domain of Pax-3 and Pax-7 proteins generating isoforms with increased affinity to target DNA sequences (Vogan et al., 1996). For the Pax-8, at least seven splice variants have been identified that do not differ in DNA binding activity but demonstrate distinct transactivation properties. Moreover, changes in the relative abundance of different splice variants during embryogenesis indicate that alternative splicing may be developmentally regulated (Kozmik et al., 1993; Poleev et al., 1992; Poleev et al., 1995).

Although several in vitro studies have demonstrated differences in the biochemical properties of Pax splice variants possible consequences of splicing on developmental or physiological functions of Pax gene products remain however elusive. We recently reported the isolation of cDNAs encoding nine Xenopus Pax-2 isoforms (Heller and Brändli, 1997). During Xenopus embryogenesis, expression of Pax-2 was confined to the nervous system, sensory organs, visceral arches, and the developing pronephric kidney. Although expression of single splice variants was temporally regulated, alternative splicing of Pax-2 transcripts does not occur in a tissue-specific manner. In this study, we analyzed the in vivo properties of Xenopus Pax-2 isoforms using gain-of-function experiments. We report here that overexpression of single Pax-2 splice variants had distinct effects on kidney organogenesis and MHB development, respectively. Our results indicate that distinct domains of the Pax-2 protein are necessary for Pax-2 gene function during development of the kidney and the MHB.
5.3.3. Results

Injection of high doses of Pax-2 RNA causes early embryonic lethality in Xenopus laevis

Several cDNAs encoding splice variants of Xenopus Pax-2/5/8 gene family (Fig. 21) have been previously isolated and characterized (Heller and Brändli, 1997; 1999). Overexpression of Pax-2 variants in Xenopus embryos was chosen as a strategy to assess their role in neural development and pronephric kidney organogenesis.

The synthesis of Pax-2 proteins from the cloned cDNAs was verified by in vitro transcription-and-translation experiments with rabbit reticulocyte lysates. Analysis of $^{35}$S-labeled translation products by SDS-polyacrylamide gel electrophoresis and autoradiography revealed Pax-2 proteins of the expected sizes (Fig. 22A, B). These results also confirm that Pax-2 cDNAs retain their original reading frames despite being subjected to extensive alternative splicing.

Overexpression studies were performed to assess whether elevated protein levels of individual Pax-2 isoforms have the ability to induce the formation of pronephric or neural tissues at ectopic sites in the developing embryo. In vitro generated transcripts of Pax-2 isoforms were injected in one blastomere of a 2-cell stage embryo, while the other blastomere remained uninjected and, thus, served as an internal control. RNA encoding nuclear β-galactosidase (nucβgal) was coinjected to follow the distribution of exogenous Pax-2 RNAs.

Dose-response experiments revealed that injecting Pax-2 RNA in amounts higher than 1 ng resulted in an arrest of embryonic development with most embryos dying shortly after gastrulation. In affected embryos the ectodermal cell layer began to detach during gastrulation. Reducing the amount of RNA injected increased survival. The observed phenotypes ranged from severely crippled embryos with defects such as spina bifida, trunk shortening and tissue thickenings in head and trunk regions (Fig. 23A, B) to normal, apparently undisturbed embryos. For each of the nine Pax-2 splice variants, a suitable dose was determined which allowed development of at least a third of the injected embryos without gross abnormalities. Interestingly, the amounts of RNA fulfilling this criterion ranged from 50 pg for Pax-2(2) to 800 pg for Pax-2(3) and Pax-2(9). We currently do not know what causes these differences, however several possibilities exist. The differences could be attributed to variations in stability and/or translation efficiencies of the injected Pax-2 RNAs.
### Results

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Fig. 21. Schematic representation of *Xenopus* Pax-2 and Pax-5 isoforms. The structure of a prototypic vertebrate Pax-2/5/8 protein is shown schematically with the corresponding domain organization (PD, paired domain; OP, octapeptide; HD, partial homeodomain) indicated. The positions of introns are indicated by arrows. Exons numbered from 1 to 10 according to Pfeffer et al. (1998) are present in all Pax-2/5/8 members, whereas the suffix '.1' in 5.1, 7.1, 8.1 refers to less well conserved, non-canonical exons. Below, cDNAs encoding different splice variants of *Xenopus* Pax-2 and the *Xenopus* Pax-5(1) variant are drawn diagrammatically. The exon composition of each splice variant is indicated with numbers. In *Xenopus*, exon 5.1 is not present in any of the Pax-2/5/8 splice variant described to date.
Fig. 22. *In vitro* translation of Pax-2 and Pax-5 splice variants. *In vitro*-coupled transcription-and-translation experiments were performed using 1 μg of the expression constructs and rabbit reticulocyte lysates in the presence of 35S-methionine. Equal volumes of translation products were loaded and separated by SDS-polyacrylamide gel electrophoresis on 7.5% (A) or 10% (B) gels. Radiolabeled proteins were visualized by autoradiography. The migration positions of size standards are shown on the left of the autoradiographs. Arrowheads indicate the front in each gel.

Certain Pax-2 proteins may also be more susceptible to degradation than others. Alternatively, the differences may reflect true differences in potency of distinct Pax-2 isoforms to interfere with early development. Injected embryos were allowed to develop to stages 30-32. Analysis of the embryos by whole mount *in situ* hybridization with probes for tissue-specific marker genes was done on embryos with normal or mild external phenotypes. We restricted our analysis to markers of the MHB primordium and the pronephric kidney.
Fig. 23. Effects of misexpressing high amounts of Pax-2 on embryonic development. One blastomere per 2-cell stage embryo was coinjected with RNA encoding Pax-2(2) (100 pg; A, B) and the lineage tracer nuclear \( \beta \)-galactosidase (nuc\( \beta \)gal; 100 pg). Injected embryos were allowed to develop to stage 30, fixed, stained for \( \beta \)-galactosidase activity, and processed for in situ hybridization using Lim-1 as a probe. Lateral views of embryos with anterior to the left are shown. (A) Severely crippled embryo with head defects and shortened trunk. Tissue thickenings in the head region are indicated (arrowheads). The expression of Lim-1 in the brain is disrupted (arrow). (B) Embryo with normal head structures and tissue thickenings in the trunk region (arrowhead). Staining for Lim-1 in the pronephric duct region is perturbed (arrow). Note that tissue thickenings in both embryos are strongly positive for nuc\( \beta \)gal staining (pale blue). (C) Control stage 30 embryo hybridized with a Lim-1 antisense probe. Abbreviations: brain (br), spinal cord (sc), pronephros (pn), and pronephric duct (pd).
Overexpression of Pax-2 isoforms alters En-2 and Pax-5 expression at the MHB

Pax-2 is one of the earliest genes known to be expressed in the region of the prospective MHB during the regionalization of the neural plate into distinct brain territories (Joyner et al., 1996; Heller and Brändli, 1997). We determined the effect of ectopic expression of different Pax-2 splice variants by monitoring the expression of genes such En-1, En-2, Pax-5, Wnt-1, and FGF-8. These genes are suitable markers for MHB formation as they are implicated in early mesencephalon and metencephalon patterning. They are expressed in spatially and temporally restricted, but overlapping patterns in the presumptive region of the MHB during early Xenopus development (Eizema et al., 1993; Hemmati-Brivanlou et al., 1991; Heller and Brändli, 1999; Christen and Slack, 1997; Wolda et al., 1993).

All nine Pax-2 isoforms were tested in overexpression experiments for their potential to affect the expression of the En-2 gene at the MHB. Injected embryos were allowed to develop to stages 30-32, and were then analyzed by in situ hybridization. We found that a subset of Pax-2 isoforms caused specific alterations of the En-2 expression domain. The observed phenotype was characterized by an expanded En-2 expression at the MHB. In addition, patches of ectopic En-2 positive cells could be detected in the vicinity of the MHB, but never at more distant locations (Fig. 24). Injection of RNA encoding either Pax-2(1), Pax-2(2), Pax-2(3) or Pax-2(4) was necessary to elicit the MHB phenotype, which occurred at incidences ranging from 49-74% (Fig. 24; Table 5). We currently do not know whether the two phenotypes, expansion and ectopic cells, are the results of one and the same process, or whether they are caused by two separate processes affected by Pax-2 overexpression. We therefore analyzed injected embryos at earlier stages (15/16 and 24), but were unable to detect abnormal En-2 expression (data not shown). These results indicate that the phenotype becomes first evident in tailbud stage embryos. Overexpression of isoforms Pax-2(5) and Pax-2(6) also resulted in an expansion of the En-2 expression domain at the MHB, but in no case ectopic En-2 expressing cells could be detected (Table 5; data not shown). Interestingly, Pax-2(5) and Pax-2(6) differ from Pax-2(1) and Pax-2(3), respectively, only by the absence of exon 5. Finally, the analysis of Pax-2(7), Pax-2(8) or Pax-2(9) indicated that these isoforms were unable to cause any significant alterations of En-2 expression at the MHB (Table 5). As En-2 expression was unaltered, we conclude that these short Pax-2 isoforms are unlikely to act in a dominant negative manner. Taken together, our results
Results

indicate Pax-2 isoforms require the presence of exons 5, 6, and 7 to elicit the full MHB phenotype (En-2 expansion and ectopic cells), while the non-canonical exons 5.1, 7.1, and 8.1 appear to be dispensable.

Fig. 24. Effects of Pax-2 overexpression on the expression of MHB marker genes. One blastomere per 2-cell stage embryo was injected with RNA encoding Pax-2(1) (400 pg; B), Pax-2(2) (50 pg; D, N, P), Pax-2(3) (800 pg; F, J, L), or Pax-2(4) (400 pg; H). 100 pg of the lineage tracer nucβgal was coinjected. Embryos were allowed to develop to stage 30, fixed, processed for β-galactosidase activity and in situ hybridization. Embryos were hybridized for the MHB markers En-2 (A-L) and Pax-5 (M-P), respectively. Lateral views of the control (A, C, E, G, I, K, M, O) and injected sides (B, D, F, H, J, L, N, P) of embryos are shown. Patches of ectopic En-2 or Pax-5 positive cells present in the vicinity of the MHB are indicated by arrowheads. Moderate expansion of the En-2 expression domain at the MHB is illustrated in B, J, and L.

We next assessed whether overexpression of Pax-2 might alter the expression of other MHB makers such as Pax-5, En-1, Wnt-1 and FGF-8. The injections were restricted to isoforms Pax-2(1) and Pax-2(2), and embryos were analyzed at stage 30. No alterations of gene expression could be detected in embryos hybridized with probes to En-1, Wnt-1 and FGF-8 (data not shown). However, a similar phenotype as observed when probing for En-2 expression could be detected in embryos analyzed for Pax-5. Expression of Pax-5 at the
MHB was enlarged on the injected side of the embryo and ectopic Pax-5 positive cells were detected in the vicinity of the MHB (Fig. 24; data not shown). This phenotype were observed with Pax-2(1) and Pax-2(2) at frequencies of 16% (n=38) and 39% (n=60), respectively.

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<tr>
<th>Experiment</th>
<th>RNA</th>
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<td>49</td>
<td>79</td>
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</table>

Synthetic RNA was injected into one blastomere of two-cell stage embryos. Nuclear β-galactosidase (nucβgal) RNA (100 pg) was coinjected as a lineage tracer. Embryos were allowed to develop to stages 30-32, fixed, stained for nucβgal, and processed for in situ hybridization with En-2. The injected sides of embryos were scored for abnormal En-2 expression which was defined as expanded and/or ectopic En-2 expression at the MHB. Expression of En-2 at the MHB was unaltered in embryos expressing nucβgal RNA (1 ng) alone. A minimum of two independent experiments were carried out for every injection.

The expression of Pax-2 in other parts or the developing central nervous system, such as the hindbrain and interneurons of the spinal cord (Burrill et al., 1997; Heller and Brändli, 1997), prompted us to examine whether Pax-2 might have a role in vertebrate neurogenesis. We chose to analyze whether overexpression of Pax-2(2) might result in ectopic neurogenesis by assaying manipulated embryos for expression of neuronal transcripts such as N-CAM, Sox-3, and neural β-tubulin (Kintner and Melton, 1987; Richter et al., 1988; Gawantka et al., 1998). Analysis of embryos provided however no evidence for ectopic induction of neurogenesis in embryos overexpressing Pax-2 (data not shown).
Overexpression of Pax-2 results in ectopic expression of the pronephric marker gene Lim-1

Loss of Pax-2 gene activity disrupts kidney organogenesis early on at the step of pronephric kidney formation which has led to the proposal that Pax-2 acts as a master regulator of kidney organogenesis (Torres et al., 1995; Brand et al., 1996; Favor et al., 1996). We decided to test this notion by assessing whether misexpression of Pax-2 might induce ectopic kidney formation. Further, we were interested in determining the consequences of Pax-2 overexpression on pronephric kidney organogenesis.

Initially, we examined the effects of Pax-2(1) overexpression by injecting single blastomeres of 2-cell stage embryos and analyzing the resulting embryos by whole mount in situ hybridization once they reached stages 30-32. Lim-1 was chosen as a marker of the pronephric lineage (Taira et al., 1994). Comparison of control and injected sides of embryos revealed massively expanded Lim-1 expression affecting both the pronephros and the pronephric ducts in approximately 50% (n=88) of the injected embryos (Fig. 25). The altered Lim-1 expression pattern suggested that Pax-2(1) promoted the formation of additional pronephric tissue which may assemble to form enlarged pronephric tubules and multiple ectopic pronephric ducts. Occasionally, Pax-2(1) overexpression led to a less dramatic phenotype with abnormal branching of the pronephric ducts and/or additional small patches of cells expressing Lim-1 ectopically in the vicinity to the developing pronephric kidney (Fig. 25D). Interestingly, the expression of Lim-1 in the central nervous system remained unaltered indicating that overexpression of Pax-2(1) had primarily an effect on the developing pronephric kidney.

We tested next whether overexpression of one of the other Pax-2 isoforms may cause similar alterations of pronephric Lim-1 expression as observed with Pax-2(1). Indeed, all Pax-2 isoforms were found to promote an expansion of the pronephric Lim-1 expression. However, the effects seen were less pronounced and occurred at considerably lower incidences (usually 2-10% of injected embryos; data not shown). Pax-2(9) represented, however, an exception in that the pronephric phenotype occurred as prominently and with comparable incidences (50%, n=86) as in embryos injected with Pax-2(1) (Fig. 25). Taken together, our findings indicate that a minimal Pax-2 protein consisting of the DNA-binding paired domain and exons 8, 9, and 10 is sufficient to induce in overexpression experiments the pronephric Lim-1 expression phenotype.
**Results**

**Fig. 25.** Effects of Pax-2 overexpression on pronephric kidney development. One blastomere per 2-cell stage embryo was injected with RNA encoding Pax-2(1) (400 pg; B, D) or Pax-2(9) (800 pg; F). The lineage tracer nucβgal was coinjected at 100 pg. Embryos were allowed to develop to stages 30/31, fixed, and processed for β-galactosidase activity and in situ hybridization. Lateral views of control (A, C, E) and injected sides (B, D, F) of embryos hybridized for Lim-1 expression and stained for nucβgal (pale blue). The expression domain of Lim-1 in the pronephros (pn) and in the pronephric duct (pd) region is enlarged, whereas the expression pattern in the central nervous system is indistinguishable from controls. In some embryos, branching of the pronephric duct (arrowhead) can be detected. Occasionally, small patches of cells expressing Lim-1 ectopically can be observed in the vicinity of the developing kidney (arrow).

*Overexpression of Pax-2 targeted to the region of the developing pronephric kidney indicates an early role for Pax-2 gene function*

We modified our original injection strategy by performing experiments in 8-cell stage rather than in 2-cell stage embryos. Lineage tracing experiments
have established that the progeny of V2 blastomeres contribute predominantly to dorsal and ventral parts of the somites, the proctodeum, and the developing pronephric kidney (Huang et al., 1998). Injection of V2 blastomeres will therefore allow more efficient targeting of Pax-2 overexpression to the region of the developing pronephric kidney, while reducing the possibility of affecting the development of other tissues of the embryo in an adverse manner. V2 blastomere injections were performed with Pax-2(9) and the lineage tracer nucßgal. At stages 30-32, survival of embryos was typically at >95% and they appeared normal upon external inspection. β-galactosidase staining of injected embryos confirmed proper targeting of the injected RNA to somites and the pronephric regions (Fig. 26). In situ hybridization of injected embryos revealed massively enlarged Lim-1 expression in the developing pronephros (Fig. 26A, B). This phenotype was observed with 75% in a high proportion of the injected embryos (Table 6). V2 blastomere injections of Pax-2(1) caused a similar Lim-1 phenotype at comparable incidences (data not shown). It therefore appears that V2 blastomere injections reproduce faithfully the pronephric phenotype initially observed after injection of Pax-2 RNAs into two-cell stage embryos (compare Fig. 25 and 26).

Recent studies have established novel markers with expression in the developing pronephric kidney. These include the antigen recognized by the monoclonal antibody 3G8 (Vize et al., 1995), and the developmental control genes Delta-1 (S. Eid and A. W. Brändli, unpublished observation), c-Ret (P. Good, unpublished observation), Pax-8 (Heller and Brändli, 1999), and WT-1 (Carrol and Vize, 1996). We performed injection experiments to investigate how Pax-2(9) overexpression affects the expression of these markers. While performing control injections with the lineage tracer nucßgal, we noticed that preferentially targeting of exogenous RNA to either the pronephros or the pronephric duct region could be achieved by placing the site of injection within V2 to the half giving rise either to V22 or V21 blastomeres, respectively (data not shown). Using this modification of the V2 blastomere injection strategy, we investigated the effects of Pax-2(9) overexpression on marker gene expression in the region of the pronephros in embryos of stages 30-32. As shown in Fig. 26, these experiments generated a phenotype comparable to the one seen in embryos analyzed for Lim-1 expression. The region of the developing pronephric tubules was significantly expanded as illustrated by the enlarged pronephric expression domains of the marker genes. As expected, the marker gene expression in the pronephric duct remained largely unaltered.
Results

Fig. 26. Effects of targeted Pax-2 expression on the pronephros of stage 30-32 embryos. One V2 blastomere per 8-cell stage embryo was coinjected with RNA encoding Pax-2(9) (400 pg) and the lineage tracer nucβgal (100 pg). Injections were performed preferentially into the dorsal region of V2 blastomeres. Embryos were allowed to develop to stages 30-32, fixed, and then processed for β-galactosidase activity and in situ hybridization. Nucβgal staining is detected in the pronephric region and in the somites (pale blue). Lateral views of the control and injected sides of embryos hybridized for Lim-1 (A, B), Pax-8 (C, D), Delta-1 (E, F), and c-Ret (G, H) expression. For all marker genes, the expression domains in the pronephros (pn) are enlarged. Furthermore, ectopic expression of c-Ret is seen occasionally in the vicinity of the pronephros (arrowhead).
Occasionally, an isolated patch of cells expressing ectopically the marker gene could be observed (Fig. 26H). Altered expression of marker genes occurred in 47-75% of the embryos injected (Table 6).

Table 6. Targeted overexpression of Pax-2 isoforms results in abnormal pronephric marker gene expression

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA</th>
<th>Amount injected (pg)</th>
<th>Marker</th>
<th>Abnormal marker expression (%)</th>
<th>No. of embryos analyzed</th>
</tr>
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<tbody>
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<td>Lim-1</td>
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</tr>
<tr>
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<td>400</td>
<td>Delta-1</td>
<td>75</td>
<td>28</td>
</tr>
<tr>
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<td>400</td>
<td>Pax-8</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>2.1</td>
<td>Pax-2(9)</td>
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<td>Pax-8</td>
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<td>52</td>
</tr>
<tr>
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<td>Lim-1</td>
<td>59</td>
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<td>32</td>
</tr>
<tr>
<td>3.3</td>
<td>Pax-2(9)</td>
<td>400</td>
<td>c-Ret</td>
<td>55</td>
<td>33</td>
</tr>
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<td>Pax-8</td>
<td>63</td>
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<td>200</td>
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<td>50</td>
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</table>

RNA encoding Pax-2 isoforms was injected into one V2 blastomere of 8-cell stage embryos. For lineage tracing, nucβgal RNA (100 pg) was coinjected. Embryos were allowed to develop to stages 30-32 (in experiments 1.1-1.3, 3.1, and 3.3) or stage 37 (experiments 2.1, 2.2, 3.2, and 4.1-4.4). They were then fixed, stained for nucβgal, and processed for whole mount in situ hybridization or immunohistochemistry. Embryos were scored for abnormally expanded marker gene expression in the region of the developing pronephric tubules. In experiments 3.1-3.3, embryos were analyzed for abnormal marker expression in the region of the pronephric duct. A minimum of two independent experiments were carried out for every injection.

The phenotype was even more pronounced when the analysis of marker gene expression was performed at later stages. Embryos at stage 37 displayed massively enlarged pronephric expression domains for Pax-8 and the 3G8 antigen (Fig. 27). Typically, the area occupied by the marker gene increased 2-5 fold in embryos overexpressing Pax-2(9). This characteristic phenotype (enlarged expression domain and occasional ectopic patches of cells) was also observed upon injection of other Pax-2 isoforms were injected (Fig. 27; data not shown).
Fig. 27. Effects of targeted Pax-2 expression on pronephric tubule formation of stage 37 embryos. One V2 blastomere per 8-cell stage embryo was injected with RNA encoding either Pax-2(1) (200 pg; B, D, I) or Pax-2(9) (400 pg; F, H). The lineage tracer nucβgal was coinjected at 100 pg. Embryos were allowed to develop to stage 37, fixed, and then processed for β-galactosidase activity. Subsequently, either in situ hybridization or immunocytochemistry was carried out. Nucβgal staining is detected in the pronephric region and in the somites (pale blue). (A-f) Lateral views of control (A, C, E) and injected (B, D, F) sides of embryos hybridized for Pax-8 expression. The expression domain of Pax-8 in the pronephros (pn) is enlarged. Occasionally, ectopic Pax-8 positive cells can be detected (arrowheads). (G, H) Lateral views of control (G) and injected (H) sides of an embryo stained with the monoclonal antibody 3G8 to detect pronephric tubules. Note the enlarged pronephros (pn) on the injected side. (I) Transversal section (70 μm) through a stage 37 embryo stained for Pax-8. The plain of section is at the level of the pronephros. On the injected side (right), the pronephros (pn) is enlarged, but properly organized into pronephric tubules (arrowheads). Pronephric tubules appear to be more numerous and have an increased diameter compared to the tubules on the control side (left).
Depending on the isoforms tested and the amount of RNA injected, the phenotype occurred in 16-63% of the injected embryos (Table 6). Vibratome sections of embryos stained in whole mount were performed to investigate the effect of Pax-2(1) overexpression on the morphology of pronephric structures. The analysis of serial sections revealed massively enlarged pronephroi on the injected side (Fig. 27I). The region occupied by the pronephric tissue was significantly enlarged creating a huge pronephric bulge which spread also into lateral regions. The pronephric tubules were prominently visible. Interestingly, the diameter of the tubules was increased by 2-5 fold in comparison to controls. The results indicated that Pax-2 overexpression promoted an increase in organ size without disrupting the differentiation of pronephric tissues.

We next asked whether Pax-2 overexpression affects other structures of the developing pronephric kidney. Overexpression experiments where Pax-2(9) RNA was targeted to the pronephric duct region caused an enlargement of
marker gene expression. Marker analysis of injected embryos included c-Ret and Lim-1 and was performed with comparable results at embryonic stages 30-32 and stage 37 embryos. (Fig. 28; data not shown). Altered marker gene expression was detected in more than half of the injected embryos (Table 6). In contrast, V2 blastomere overexpression experiments with Pax-2(9) failed to alter the expression of the glomerular marker WT-1 (data not shown). Taken together, targeted overexpression of Pax-2 isoforms revealed an expansion of the pronephric tubule and duct tissues, while other tissues such as the glomus appeared to be insensitive to Pax-2 overexpression. Furthermore, the phenotype was observed irrespective of whether early (e.g. Lim-1 and Pax-8) or late markers (e.g. the 3G8 antigen) of the developing pronephric kidney were analyzed.

**Pax-5 substitutes for Pax-2 function in overexpression experiments targeted at the developing pronephric kidney**

The vertebrate Pax-2/5/8 gene family appears to have arisen from a single ancestral gene through two genome duplications which occurred early in vertebrate evolution (Noll, 1993; Pfeffer et al., 1998; Heller and Brändli, 1999; Kozmik et al., 1999). Expression of Pax-2 and Pax-8, but never Pax-5, is associated with kidney organogenesis as studies in different vertebrate species have demonstrated. Since Pax-5 proteins share the same structural organization and a high degree of similarity with Pax-2 and Pax-8, we asked whether Pax-5 might be able to substitute for Pax-2 in overexpression experiments. Sequence analysis of the five known Xenopus Pax-5 splice variants has demonstrated that the original reading frame remains only for Pax-5(1) unaltered by alternative splicing (Heller and Brändli, 1999). The exon composition of Pax-5(1) and Pax-2(1) has been conserved (Fig. 21).

We performed V2 blastomere injections as described above with Pax-5(1) RNA. Embryos were reared to stages 30-32 or 37, fixed, and processed for in situ hybridization to visualize the expression of the pronephric marker genes Pax-2, Pax-8, Lim-1, and Delta-1 (Fig. 29; Table 7; data not shown). Inspection of stage 30-32 embryos revealed significantly enlarged areas of Lim-1 and Delta-1 expression in the region of the developing pronephric tubules (not shown). This phenotype was seen in at least 30% of the injected embryos (Table 7). Analysis of stage 37 embryos indicated that both the expression of pronephric tubule as well as duct markers was expanded as a response to Pax-5(1) overexpression (Fig. 29; Table 7). The pronephric phenotypes induced by
Results

overexpression of Pax-5(1) were indistinguishable from those observed after Pax-2 isoform. Our findings demonstrate that Pax-5(1) can functionally substitute Pax-2(2) in vivo, providing further evidence for functional redundancy among Pax-2/5/8 genes.

Fig. 29. Effects of targeted Pax-5 expression on the pronephric kidney of stage 37 embryos. One V2 blastomere each per 8-cell stage embryos was co injected with RNA encoding Pax-5(1) (100 pg) and the lineage tracer nuclear nucβgal (100 pg). Embryos were allowed to develop to stage 37, fixed, and then processed for β-galactosidase activity and in situ hybridization. (A, B) Lateral views of control and injected sides of an embryo hybridized for Pax-8 expression. The expression domain of Pax-8 in the pronephros (pn) is enlarged. (C, D) Lateral views of control and injected sides of an embryo hybridized for Pax-2 expression. Again an enlargement of the Pax-2 expression domain in the pronephros (pn) can be observed. (E, F) Lateral views of control and injected sides of an embryo hybridized for Lim-1 expression. The expression domain of Lim-1 in the pronephros (pn) as well as in the pronephric duct (pd) region is enlarged.
RNA (100 pg) encoding Pax-5(1) was injected into one V2 blastomere of 8-cell stage embryos. For lineage tracing, nucßgal RNA (100 pg) was coinjected. Embryos were allowed to develop to stage 30-32 (experiments 1.1 and 1.2) or stage 37 (experiments 2.1-2.3). They were fixed, stained for nucßgal, and processed for in situ hybridization. Embryos were scored for abnormally expanded marker gene expression in the region of the developing pronephric tubules. In experiment 2.3, embryos were analyzed for abnormal marker expression in the region of the pronephric duct. A minimum of two independent experiments were carried out for every injection.

**Table 7. Overexpression of Pax-5 results in abnormal pronephric marker gene expression**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>RNA</th>
<th>Marker</th>
<th>Abnormal marker expression (%)</th>
<th>No. of embryos analyzed</th>
</tr>
</thead>
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<td>Lim-1</td>
<td>33</td>
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</tr>
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<td>Delta-1</td>
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<td>61</td>
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<td>Pax-8</td>
<td>24</td>
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</tr>
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<td>Pax-5(1)</td>
<td>Lim-1</td>
<td>32</td>
<td>25</td>
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</tbody>
</table>

*Animal cap cultures overexpressing Pax-2 isoforms develop large oedema which may indicate a role in pronephric tubule induction*

In *Xenopus*, animal cap cultures treated with the growth factor activin represent an attractive model to study the induction of mesodermal tissues outside the context of an entire organism (Asashima, 1994; Dawid, 1994; Kessler and Melton, 1994; Slack, 1994). Interestingly, however, animal cap cultures treated with activin do not generate pronephric tissues. Induction of pronephric tubule formation can however be achieved by treating animal caps with activin and retinoid acid (Moriya et al., 1993; Uochi and Asashima, 1996).

We initiated studies to investigate the effects of Pax-2 overexpression on animal cap cultures and to assess whether Pax-2 is sufficient to promote pronephric tubule formation in activin-treated animal cap explants. Overexpression of Pax-2 was achieved by injecting into the animal pole region of both blastomeres of 2-cell stage embryos. RNA encoding either Pax-2(1) or Pax-2(9) in combination with the lineage tracer nucßgal were used for the injections. Embryos injected with nucßgal alone served as controls. Animal caps were cut once embryos reached stage 8-9 and cultured in the presence of activin. Control cultures were prepared without activin. Animal caps were cultured until control embryos reached stage 37 and then subjected to visual
Results

Animal cap cultures expressing nucβgal alone and treated with activin contained strongly elongated explants with little pigmentation (data not shown). The addition of retinoic acid to these cultures suppressed elongation and generated explants with fluid-filled oedema (Fig. 30). Pax-2 injected explants cultured in absence of any factors added were indistinguishable from cultures prepared from uninjected embryos (data not shown). Interestingly, however, when activin was included with animal cap explants overexpressing Pax-2 isoforms the resulting phenotype strongly resembled the cultures treated with activin and retinoic acid (Fig. 30). Typically, oedema could be observed in the majority of the cultures. The explants differed significantly only in the pigmentation indicating that Pax-2 may have a role in pigment cell development. Though preliminary, our findings provide evidence that Pax-2 overexpression may induce or promote the formation of pronephric tissue in activin-treated animal cap cultures.

Fig. 30. Effects of Pax-2 overexpression on animal cap explants. Both blastomeres of 2-cell stage embryos were injected into the animal hemisphere with RNA encoding either Pax-2(9) (400 pg; B) or Pax-2(1) (200 pg; C). The lineage tracer nucβgal was coinjected at 100 pg. Injected and uninjected embryos (control) were allowed to develop to stage 8-9, at which animal caps were dissected. Injected animal caps were treated for 12 h at 22°C with activin (5 ng/ml) alone. Control explants were cultured in the presence of activin (5 ng/ml) and retinoic acid (100 μM). Injected animal caps were fixed and processed for β-galactosidase activity once reference embryos reached stage 37 (pale red staining). (A) Control explants treated with activin and retinoic acid show characteristic oedema formation associated with pronephric tubule induction (arrows). (B, C) Oedema (arrows) and patches of pigmented cells (arrowheads) are detected with Pax-2 injected animal cap cultures treated with activin alone.
5.3.4. Discussion

Despite a large effort directed at identifying molecular mechanisms controlling early steps of vertebrate development they are still poorly understood. The transcription factors of the Pax gene family have been shown to play important roles in embryonic development and organogenesis (Dahl et al., 1997; Mansouri et al., 1996). Of the nine Pax gene family members, we focused our analysis on Pax-2 which is expressed in the nervous system, the sensory organs, the visceral arches, and the excretory system of the developing embryo. We show here that Pax-2 plays central roles in regulating neural development and kidney organogenesis.

Pax-2 acts early in the genetic cascade regulating kidney organogenesis

In the present study, we examined the effects of Pax-2 overexpression on early kidney development in Xenopus embryos to elucidate the role of Pax-2 isoforms. Judged by morphology and analysis of marker gene expression, ectopic Pax-2 expression resulted in a massive enlargement of pronephric tissue. Remarkably, the enlarged pronephros is properly organized into pronephric tubules of increased diameter. A morphological analysis of semi-thin plastic sections will have to be performed and may reveal whether also a change in pronephric tubule number had occurred. Expansion of the expression domain of early and late pronephric marker genes like Lim-1, Pax-8, Delta-1, c-Ret and the 3G8 antigen indicates that Pax-2 acts early in the molecular cascade controlling kidney development. Our observations provide further compelling evidence for Pax-2 as a central regulator of kidney organogenesis.

Multiple pathways in early MHB development

We performed Pax-2 gain-of-function experiments to also assess the role of Pax-2 in the development of the MHB. Overexpression of Pax-2 led to an enlargement of the expression domains of MHB markers. Furthermore, ectopic cells positive for MHB marker genes were detected in vicinity to the MHB. Interestingly, we found that only two of the five marker genes used to analyze the effects of Pax-2 overexpression revealed phenotypes at the MHB. While enlarged and ectopic En-2 and Pax-5 expression could be observed, Wnt-1, En-1 and FGF8 expression was unaltered by injection of Pax-2 transcripts. These findings may either suggest that several Pax-2 independent pathways
Results

exist or that Pax-2 is further downstream in the genetic cascade controlling MHB development.

Initiation of Wnt-1 and Pax-2 expression in the prospective MHB occurs at similar timepoints during *Xenopus* development (N. Heller and A.W. Brändli, unpublished observation). In the mouse, Wnt-1 appears to precede Pax-2 expression (Joyner, 1996). The latter finding indicates that Wnt-1 transcription is initiated independently from Pax-2 and may explain why its expression is not affected by overexpression of Pax-2. Functional studies however have so far failed to provide evidence for the control of Pax-2 expression by Wnt-1. In mice, a homozygous mutation for Wnt-1 does not affect Pax-2 expression (McMahon and Bradley, 1990; Rowitch and McMahon, 1995). Furthermore, Wnt-1 misexpression in zebrafish does not activate Pax-2 expression at least at early embryonic stages (Kelly and Moon, 1995). Therefore, Pax-2 and Wnt-1 appear to be components of different, independent signaling cascades determining events in MHB development.

Our findings indicating Pax-2 as a regulator of En-2 and Pax-5 expression are consistent with results obtained through the analysis of the zebrafish mutant *noi*, a Pax-2 loss-of-function mutant (Lun and Brand, 1998; Pfeffer et al., 1998). Whereas the expression of En-2 and Pax-5 is lost, Wnt-1 and FGF-8 are unaltered in homozygous *noi* mutant embryos. The results from gain- and loss-of-function studies therefore indicate that Pax-2 controls En-2 and Pax-5 expression, but that additional Pax-2 independent signaling pathways may operate in parallel to induce gene expression of early markers of MHB, such as FGF-8 and Wnt-1.

**Possible roles of Pax-2 in kidney organogenesis and MHB development**

Overexpression of Pax-2 led to the expansion of the expression domains both of pronephric kidney as well as MHB markers. We are currently unable to distinguish between a simple expansion of the affected tissue or an incomplete separation of distinct, ectopic regions with pronephric or neural fate. We therefore cannot resolve conclusively the origin of the additional cells expressing the marker genes. In principle, several mechanisms can account for the enlargement of the MHB region and the pronephric kidney observed in embryos overexpressing Pax-2. These include:

1. altering cell fate in neighboring cell populations,
2. increased or prolonged proliferation rates, and
3. inhibition of apoptotic events.
Further, it is conceivable that a combination of the above mentioned possibilities may be occurring. We will discuss now the available evidence for these different possibilities in greater detail.

_Evidence for a role of Pax-2 in cell fate determination_

A potential to determine cell fate has been shown for several members of the Pax gene family. Overexpression of Pax-3 in neural tube explants leads to the activation of muscle differentiation markers in neuroepithelial cells. In contrast, transient expression of Pax-3 in fibroblasts fails to elicit myogenesis (Maroto et al., 1997). Ectopic eye structures are induced on the wings, the legs and the antennae in *Drosophila* after expression of the eyeless gene, a homolog to Pax-6, in various imaginal disc primordia (Halder et al., 1995). In *Xenopus*, the formation of ectopic lenses restricted to the anterior region of the embryo can be induced by overexpression of Pax-6 (Altmann et al., 1997). Pax-3 and Pax-6 have therefore the potential to direct cell fate towards skeletal muscle and eye development, respectively, but these functions depend on the presence of competent tissue.

In the present study, we show that overexpression of Pax-2 is not sufficient to induce the formation of ectopic kidneys or pronephric tissue at distant locations in the embryo. Injection of Pax-2 isoforms is however sufficient to induce ectopic transcription of pronephric marker genes within the region of the intermediate mesoderm. Both enlargement of the pronephros as well as broadening or branching of the pronephric duct was observed. Similarly, cells positive for marker genes of MHB development are detected at ectopic locations in the vicinity of the MHB. These findings indicate that Pax-2 might have the ability to change cell fate towards a nephric or neural fate in very defined locations of the embryo. Our preliminary results with animal cap explants where Pax-2 requires the presence of competent tissue to drive cell fate changes are in support of this view. Pax-2 injected animal cap cultures are only able to form oedema, an indicator of pronephric tubule induction (Moriya et al., 1993), if treated with activin. Taken together, the phenotypes of Pax-2 injected embryos and animal cap explants suggest that Pax-2 may be able to specify cells of mesodermal origin towards pronephric fates.

If Pax-2 acts in the intermediate mesoderm to drive cells into pronephric cell fates, overexpression of Pax-2 should result in the depletion of those cell fates that are not dependent on Pax-2 function. The intermediate mesoderm gives rise to the cell lineages of the pronephric kidney, and the haematopoietic and vascular systems. In the pronephric kidney, Pax-2 expression is associated
with developing pronephric tubules and duct tissue (Heller and Brändli, 1997). Pax-2 overexpression may therefore recruit cells of blood, glomerular or stromal lineages to tubular and duct fates. Therefore, analysis of gene expression in embryos overexpressing Pax-2 should be extended to markers of these tissues, such as the GATA factors (Bertwistle et al., 1996), Xmsr (Devic et al., 1996), WT-1 (Carrol and Vize, 1996), XFD-9 and XFD-11 (Kö ster et al., 1998; Lef et al., 1996).

Evidence for a role of Pax-2 in cell proliferation control

Abnormal cell proliferation caused by elevated levels of Pax-2 proteins could present an alternative mechanism generating excessive tissue with expression of kidney and MHB marker genes. Several lines of evidence support a role of Pax-2 in cellular proliferation control:

1. The localization of Pax-2 and Pax-8 proteins to the highly proliferative zone of developing kidney (Torban and Goodyer, 1998).
2. All members of the Pax-2/5/8 class are capable of repressing transcription of the tumor suppressor gene p53 in vitro (Stuart et al., 1995).
3. Reduction of Pax-2 expression using Pax-2 antisense oligonucleotides induces a dramatic decline of the proliferation rates of renal carcinoma cells (Gnarra and Dressler, 1995).
4. NIH3T3 cells transfected with Pax-2 are able to promote contact-independent growth and tumorigenesis when injected in nude mice (Maul becker and Gruss, 1993).
5. The expression of Pax-2 and Pax-8 is abnormally upregulated in Wilms' tumor, a paediatric renal carcinoma of mesenchymal origin (Dressler and Douglass, 1992; Poleev et al., 1992).
6. Renal Pax-2 expression persists postnatally in infants with cystic dysplasia causing kidney malformations (Winyard et al., 1996). Similarly, mice overexpressing Pax-2 are born with large multicystic kidneys (Dressler et al., 1993). The presence of cysts suggests disturbed control of cell proliferation.

Differentiation of renal tubules and ducts is accompanied by down-regulation of Pax-2 expression (Dressler et al., 1990; Dressler and Douglass, 1992; Eccles et al., 1992). Abnormal persistent Pax-2 expression may therefore prolong the proliferation phase of cells within the developing pronephric kidney. Similarly, the expanded expression domain of MHB marker genes might be a consequence of the persistence of mitotically active cells in the MHB primordium caused by elevated levels of Pax-2 proteins.
Evidence for a role of Pax-2 in apoptosis

Cell number can also be controlled during embryogenesis by programmed cell death (apoptosis) (Shi et al., 1998; Wyllie et al., 1980). Therefore, inhibition of apoptotic events during kidney organogenesis has to be taken into account as a potential mechanism leading to an expansion of pronephric kidneys in Pax-2 overexpressing embryos.

In vitro studies have demonstrated that metanephric mesenchymal cells undergo programmed cell death in the absence of an inducing signal (Koseki et al., 1992). As metanephric kidney development proceeds, apoptosis appears to be a mechanism for eliminating surplus mesenchymal cells that fail to undergo differentiation. However only about 3% of the cells in the developing rat kidney display pyknotic nuclei (Coles et al., 1993). Naturally occurring cell death during early Xenopus development has been recently analyzed in whole-mount by in situ TUNEL labeling (Hensey and Gautier, 1998). Importantly, this study did not reveal any apoptotic events in the developing pronephric kidney up to stage 36. By this time, pronephric structures of Pax-2 overexpressing embryos were already abnormally enlarged. Therefore, Pax-2 mediated inhibition of programmed cell death appears to be an unlikely mechanism. Final proof will however require the comparison of in situ TUNEL labeling of control embryos with embryos overexpressing Pax-2.

Concluding remarks on the Pax-2 overexpression phenotypes

Overall, we are currently not able to attribute the observed phenotypes clearly to a defined role of Pax-2 in cell fate determination, in cell proliferation or a combination of both events. The analysis of Pax-2 deficient animals has also not been helpful to resolve this issue. For example, it is currently not known, whether pronephric tubules form in mutant animals. In Pax-2 deficient mice and zebrafish, the rudimentary pronephric ducts form, but fail to elongate (Brand et al., 1996; Favor et al., 1996; Torres et al., 1995). The transient expression of Pax-8 in the elongating pronephric duct may be sufficient to permit initiation of pronephric duct formation even in the absence of Pax-2. Therefore, it will be of interest to analyze mutants deficient in both Pax-2 and Pax-8 to establish whether Pax gene function is required at the earliest stages of kidney organogenesis. Nevertheless, if pronephric kidney organogenesis is not initiated in these mutants, it might be difficult to attribute the phenotype unequivocally to a role of Pax-2 in cell fate determination. Lack of the pronephric kidneys might be also a consequence of impaired cell proliferation.
preventing expansion of stem cell populations. Analysis of expression of very early marker genes for the pronephric lineage, e.g. Lim-1 or LFB-1, might help to distinguish between these two possibilities.

**Functional redundancy of Pax-2/5/8 proteins**

The vertebrate Pax-2/5/8 genes are believed to be derived from two independent gene duplications of a common ancestral gene (Noll, 1993). While the enhancer elements of the Pax-2/5/8 genes appear to have diverged, the coding regions are highly conserved between vertebrate species (Heller and Brändli, 1997; Heller and Brändli, 1999; Walther et al., 1991). Analysis of the DNA-binding properties of Pax-2/5/8 paired domains has failed to reveal distinct consensus sequences for DNA recognition (Adams et al., 1992; Czerny et al., 1993; Epstein et al., 1994a; Zannini et al., 1992). Furthermore, all three Pax-2/5/8 proteins were found to have similar affinities for DNA binding sites identified in the promoters of the En-2 and p53 genes (Song et al., 1996; Stuart et al., 1995). Therefore, Pax-2/5/8 may display functional redundancy in the transcriptional regulation of target genes in vivo. Analysis of mutant phenotypes of mice either deficient for Pax-2, Pax-5 or Pax-8 suggest compensatory functions of these proteins in tissues that express more than one Pax-2/5/8 gene during vertebrate embryogenesis (Mansouri et al., 1998; Schwarz et al., 1997; Urbánek et al., 1994). Recently, we provided direct evidence that Pax-2 and Pax-8 can substitute each other in vivo (Heller and Brändli, 1999). Our results revealed that Pax-2 is associated with thyroidogenesis in *Xenopus* embryos, whereas the mammalian thyroid gland expresses Pax-8 instead (Plachov et al., 1990). Furthermore, analysis of homozygous mice deficient for both Pax-2 and Pax-5 revealed severe midbrain and cerebellar defects whereas single mutants display only mild brain abnormalities indicating functional redundancy (Schwarz et al., 1997). In the present study, we found that overexpression of Pax-5 which is normally absent in the developing kidney resulted in an expansion of the expression domains of pronephric kidney marker genes. As a similar phenotype was observed in embryos injected with Pax-2 RNA, we conclude that Pax-5 has the capability to mimick Pax-2 function during pronephric kidney organogenesis. We believe that also overexpression of Pax-8 would elicit a similar phenotype. Altogether, our data are consistent with the notion of functional redundancy of Pax-2/5/8 family members in vivo.
Pax-2 isoforms display functional differences in kidney organogenesis and MHB development

A further objective of this study was to identify and characterize the activities of individual Pax-2 isoforms. Pax proteins act as transcription factors harboring distinct domains for recognition of target genes and transcriptional activation. Alternative splicing has been found to affect both aspects of Pax protein function in vitro (Dehbi et al., 1996; Epstein et al., 1994b; Kozmik et al., 1993; Poleev et al., 1995; Vogan et al., 1996; Zwollo et al., 1997). Here, overexpression of nine different Pax-2 splice variants with subsequent analysis of marker gene expression confirms an effect of alternative splicing on Pax-2 protein function in vivo.

Overexpression of Pax-2 isoforms affected the expression of marker genes to a variable degree. Pax-2(1) and Pax-2(9) induced alterations of pronephric marker gene expression with the highest incidence of all nine Pax-2 isoforms. Interestingly, Pax-2(9) is the shortest splice variant identified to date. The exon composition indicates that Pax-2(9) consists of a DNA-binding domain and the most C-terminal exons 8, 9, and 10. These exons are predicted to act as a minimal transactivation domain (Dörfler and Busslinger, 1996). Our findings with Pax-2(9) indicate that a Pax-2 protein consisting of a DNA-binding domain and the most C-terminal exons is sufficient to elicit a pronephric phenotype in overexpression experiments. In contrast, Pax-2 proteins with additional exons were necessary to induce effects of the MHB. Ectopic activation of En-2 expression required the presence of exons 5, 6, and 7. Pax-2 isoforms lacking these exons had no significant effect on En-2 expression, while isoforms only deficient of exon 5 displayed a weak alteration of En-2 expression at the MHB. Together, these observations provide the first evidence for distinct functions of Pax-2 isoforms in vivo.

Distinct Pax-2 isoforms act in a tissue-specific manner

Alternative splicing of Pax-2 transcripts is subjected to temporal regulation during Xenopus embryogenesis. The analysis of dissected embryonic heads and pronephric kidneys could however not provide evidence for tissue-specific splicing (Heller and Brändli, 1997). Nevertheless, we detected in the present study that overexpression of single Pax-2 isoforms resulted in tissue-specific phenotypes.

How can Pax-2 isoforms generate distinct, tissue-specific activities although they appear to be present in all examined tissues? It is currently not
known whether translation of Pax-2 transcripts occurs in a tissue-specific manner. The expression of different Pax-2 isoforms could be regulated at the translational level. Antibodies raised against specific domains of Pax-2 isoforms may therefore be a valuable tool to clarify this issue. Alternatively, the function of the different Pax-2 isoforms could be modulated by additional factors present or active only in specific tissues. These tissue-specific factors may interact with those Pax-2 isoforms harboring the appropriate binding domain to initiate transcription of specific target genes. To date, no direct experimental evidence exists in support of this hypothesis. A yeast two-hybrid screen could however be employed to identify candidate proteins. Overall, our results reveal that alternative splicing serves to increase the functional diversity of Pax-2 genes during development. In the present study, we have been able to gain first insights into the functional specificity of the different Pax-2 isoforms during MHB and kidney organogenesis in vivo. Further experiments will have to follow in order to provide a comprehensive picture of the components acting up- or downstream of Pax-2 isoforms in the signaling cascades determining the developmental program of the different tissues. A separate focus of interest for future studies will be to understand the molecular mechanisms that help Pax-2 isoforms to achieve their tissue-specific activities.
5.3.5. Materials and Methods

Embryo collection and animal cap assay

Pigmented *Xenopus laevis* frogs were purchased from African Reptile Park (Tokai, South Africa), and albinos from Dr. Charles Thièbaud (Station de zoologie expérimentale, Université de Genève, Chêne-Bougeries, Switzerland). *Xenopus* embryos were obtained by *in vitro* fertilization as described by (Newport and Kirschner, 1982) and staged according to (Nieuwkoop and Faber, 1956). Dissection of animal caps and culture conditions are described in (Heller and Brändli, 1997; Heller and Brändli, 1999).

Plasmids and constructs

The isolation of two *Xenopus* Pax-5 cDNAs [Pax-5(1), Pax-5(2)] and nine different *Xenopus* Pax-2 cDNAs [Pax-2(1)-(9)] had been previously reported (Heller and Brändli, 1997; Heller and Brändli, 1999). Each Pax-2 cDNA was restriction enzyme digested with EcoRI and Xbal from the Bluescript SKII+ vector. The Pax-5(1) and Pax-5(2) cDNAs were restriction enzyme digested with Xbal and Xhol. cDNA fragments were subcloned into the pCS2+ expression vector that contains a CMV promoter, binding sites for SP6 and T7 polymerases, and a SV40 consensus polyadenylation site (Rupp et al., 1994; Turner and Weintraub, 1994). The following plasmids were constructed CS.4A3, CS.7A3, CS.10A8, CS.4, CS.32, CS.29, CS.5*, CS.28*, CS.30*, CS.5Pax16, and CS.5Pax15 containing the entire open reading frames of Pax-2(1) to Pax-2(9), Pax-5(1) and Pax-5(2), respectively, and were used for *in vitro* synthesis of RNA. The CS2+ vector and plasmid CS2+ßgal, a CS2+ derivative permitting synthesis of RNA encoding a fusion protein of β-galactosidase with the SV40 large T nuclear localization signal, were kindly provided by Dr. David Turner (University of Michigan, Ann Harbor, USA).

In vitro transcription and translation

*In vitro* coupled transcription and translation experiments were performed with Pax-2 and Pax-5 expression constructs to verify the synthesis of an appropriate sized protein. Messenger RNA was generated from 1 μg supercoiled DNA with SP6 RNA polymerase (Boehringer Mannheim) and translated *in vitro* using a reticulocyte lysate system (TNT, Promega) according to the manufacturer's recommendations. Translation products were labelled
Results

by including $^{35}$S-methionine in the reaction. Equal volumes of proteins were
separated by reducing 10% or 7.5% SDS-polyacrylamide gel electrophoresis.
The gels were fixed for 20 minutes in 50% methanol, 10% acetic acid and
treated with enhancer (Enhance, NEF-981G, Dupont). After drying, gels were
exposed to X-ray film (RX, Fuji) for 3 hours at -70°C.

In vitro transcription and microinjections

In vitro synthesis of capped RNA from linearized plasmids was carried out
using the MEGAscript SP6 kit (Ambion) following the manufacturer's
instructions. The ratio of cap analog (diguanosine triphosphate, G(5')ppp(5')G;
Pharmacia Biotech) to GTP was 4:1. Unincorporated nucleotides were removed
by gel filtration through G-50 Sephadex columns (Quick Spin Columns,
Boehringer Mannheim). The RNA concentration was determined
spectrophotometrically. Embryos in 2% Ficoll (Sigma) / 0.5x MMR (Newport and
Kirschner, 1982) were injected with capped RNA either at the 2-cell stage into
one or both blastomeres or at the 8-cell stage into one of the two V2
blastomeres (Moody, 1987) using a pneumatic injector PLI-100 (Medical
Systems Corp.). Injected embryos were transferred 1h after injection to 0.1x
MMR and cultured at 16°C until the desired developmental stage was reached.
Embryos were co-injected with RNA encoding nuclear β-galactosidase (100-
200 pg/blastoemere) for lineage tracing purposes.

β-galactosidase staining

Embryos were fixed in MEMFA fix (Harland, 1991) for 30 minutes at room
temperature, rinsed three times with PBS and stained at 37°C in PBS
containing 5 mM K$_3$Fe(CN)$_6$, 5 mM K$_4$Fe(CN)$_6$, 1 mg/ml X-gal (5-bromo-4-
chloro-3-indolyl-β-D-galactopyranoside) and 2 mM MgCl$_2$. Once staining
reached the desired intensity, the embryos were refixed in MEMFA fix for at
least 1 hour at room temperature, rinsed with PBS and dehydrated in absolute
methanol. Stained embryos were stored at -20°C until they were used for in situ
hybridization or immunohistochemistry analysis.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out essentially according to
(Harland, 1991). The standard protocol was modified by replacing methanol
with ethanol and omitting the RNase digestion step. Antisense RNA probes
were synthesized using rUTP-digoxigenin (Boehringer Mannheim Biochemicals). Unincorporated nucleotides were removed by gel filtration through G-50 Sephadex columns (Quick Spin Columns, Boehringer Mannheim). Sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) were used to detect digoxigenin-labeled probes. The color reaction was performed using BM purple (Boehringer Mannheim). Stained wild-type embryos were fixed and bleached by exposure to bright fluorescent light in 1% H₂O₂, 5% formamide and 0.5 X SSC for 1h under influence of light. Probes and wild-type expression patterns are described elsewhere: En-1 (Eizema et al., 1994), En-2 (Hemmati-Brivanlou et al., 1991), Pax-5 (Heller and Brändli, 1999), Pax-8 (Heller and Brändli, 1999), FGF-8 (Christen and Slack, 1997), Wnt-1 (Wolda et al., 1993), Delta-1 (Chitnis et al., 1995), Lim-1 (Taira et al., 1992), Sox-3 (Gawantka et al., 1998), NCAM (Kintner and Melton, 1987), and c-Ret (P. Good, unpublished data).

**Whole mount immunohistochemistry**

Whole mount immunohistochemistry was carried out essentially according to (Klymkowsky and Hanken, 1991). Incubation with the primary antibody 3G8 (Vize et al., 1995) was performed over night at 4°C with hybridoma supernatants (1: 300 diluted in TBT / 20% horse serum). The alkaline phosphatase-conjugated secondary antibody (Vector Labs) was applied in a 1:500 dilution for 6 h at room temperature. The colour reaction was performed using BM purple (Boehringer Mannheim).

**Tissue sectioning**

Fixation of embryos, embedding in agarose and sectioning at 50-100 µm with a vibrating blade microtome has been described previously (Heller and Brändli, 1997). Images of embryos or sections were either captured with a CCD camera mounted on a Zeiss STEMI 2000-C stereoscopic microscope and processed using Adobe Photoshop Version 4.0. Photography was performed as previously described (Heller and Brändli, 1997).
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6. Perspectives

6.1. Identification of the regulatory elements conferring pronephric expression of the Pax-2 and Pax-8 genes using transgenic frogs

With the identification of Pax-8 as the earliest marker of the pronephric lineage (Heller and Brändli, 1999) and Pax-2 as an important regulator of kidney organogenesis (Torres et al., 1995; Heller and Brändli, manuscript in preparation), the question of the molecular components regulating their precise spatial expression arises. Tissue-specific gene expression is achieved by regulatory elements, promoters and enhancers, which are located in the 5'-flanking regions and in the first introns of genes. Therefore, a detailed analysis of these regions and the identification of factors binding to specific regulatory elements located within them may allow the elucidation of the genetic cascade acting upstream of Pax-2 and Pax-8 regulating kidney development. In contrast to the complex expression pattern of Pax-2, Pax-8 is only associated with the developing pronephric kidney and the otic vesicle.

I therefore propose to screen a genomic Xenopus library with a Pax-8 cDNA probe to isolate DNA sequences harboring putative promoter elements. 5'-untranslated sequences will be fused to a reporter gene, β-galactosidase or green fluorescent protein (GFP), to test whether these sequences can convey correct temporal and spatial transcriptional activation. In Xenopus, promoter analysis can be performed using two different experimental approaches. Correct developmental regulation of the expression can be determined by microinjection of plasmid encoding the chimeric genes into fertilized eggs (Gurdon and Melton, 1981; Mohun et al., 1986; Sargent and Mathers, 1991). The disadvantage of this method is that usually only a minority of cells express the transgene. The problem of mosaic expression has been recently overcome by the development of methods for transgenesis in Xenopus (Kroll and Amaya, 1996). With this method, transgenes are introduced into sperm DNA and the sperm nuclei are subsequently injected into unfertilized eggs. Tissue-specificity of reporter gene expression is assessed by staining for β-galactosidase or excitation of GFP. Promoter deletion constructs could be generated to define the minimal DNA elements directing pronephric expression and correct developmental timing. After defining the minimal DNA sequences, computer-aided sequence analysis could be performed to identify consensus motifs for transcription factor binding. Alternatively, electrophoretic mobility shift assays
(EMSA) could be employed to identify biochemically proteins with binding activities. In a next step, mutagenesis of putative binding sites could be performed to establish whether initiation and maintenance of Pax-2 expression in the pronephric kidney is altered. Again these studies would be done using transgenic *Xenopus* embryos. A similar experimental approach could be used to identify components regulating Pax-2 during pronephric kidney organogenesis or MHB development, respectively.

### 6.2. Identification of downstream targets of Pax-2 and Pax-8 in the molecular cascade controlling pronephric kidney development

For a comprehensive analysis of the molecular mechanisms underlying kidney organogenesis, the identification of downstream target genes of Pax-2 and Pax-8 is of obvious importance.

Interestingly, studies aimed at identifying genes acting in pathways leading to the establishment of the visual system have provided valuable information concerning candidate genes that may have functions also in the early developing kidney. In *Drosophila*, the Pax-6 gene *eyeless* acts early in the genetic hierarchy involved in eye development and can direct the formation of extra eyes at ectopic locations (Haider et al., 1995; Quiring et al., 1994). Recently, *sine oculis* and *eyes absent* have been identified as downstream components of *eyeless* during eye development (Halder et al., 1998). In vertebrates, four *eya* homologs (Eya-1 to Eya-4) have been identified (Abdelhak et al., 1997; Xu et al., 1997; Zimmerman et al., 1997; Borsani et al., 1998). All four Eya genes are expressed in the developing eye and in the branchial arches. Interestingly, Eya-1 and Eya-2 expression is also associated with the metanephric kidney. Recently, a deletion in the Eya-1 gene was identified underlying branchio-oto-renal (BOR) syndrome (Abdelhak et al., 1997). The deficiencies associated with BOR syndrome include hearing loss and branchial cysts, but also renal defects (Misra and Nolph, 1998). Eya-1 might therefore be a further important component in the genetic cascade underlying kidney organogenesis. A mammalian gene family with high sequence similarity to *sine oculis* has also been described recently (Oliver et al., 1995; Oliver et al., 1995). This family contains at least five members, with Six-3 probably being the murine functional homolog of *sine oculis* in the developing eye. There is strong genetic evidence indicating that Six-3 acts downstream of Pax-6 during eye development (Halder et al., 1998). Remarkably, murine Six-2 has been shown to be expressed in the nephrogenic
mesenchyme of the developing kidney (Oliver et al., 1995; Torres et al., 1995). In summary, these observations suggest that an analogous pathway involving Pax genes (Pax-2 and Pax-8), Six genes and members of the Eya gene family might act in kidney development.

We have begun to test this hypothesis using a RT-PCR strategy with degenerate primers to isolate Xenopus orthologues of the Six gene family. In a first experiment, cDNAs had been isolated that showed highest homology to Six-1 (N. Heller and A.W. Brändli, unpublished data). Whole mount in situ hybridization analysis using dioxigenin-labeled RNA probes to determine the spatial expression of this novel Xenopus Six gene revealed expression during somitogenesis and neural development. This expression pattern is very similar to that of Six-1 in mouse (Oliver et al., 1995). The clone appears therefore to encode the Xenopus Six-1 orthologue. In the future, efforts should be focussed on isolating Xenopus Six-2 cDNAs using a modification of the RT-PCR approach. Alternatively, a Xenopus cDNA library could be screened with a murine Six-2 cDNA probe under low stringency conditions to isolate Xenopus Six-2 sequences. Further studies should focus on determining the relationship between Pax-2/Pax-8 and Six-2 in the developing kidney. A similar RT-PCR approach can be used to isolate Xenopus Eya-1 and Eya-2 cDNAs.

6.3. Determination of the DNA binding and transactivation activities of Pax-2 isoforms

Analysis of in vitro DNA binding and transactivation properties of individual Pax-2 isoforms would provide information that could become useful in interpreting our functional data obtained in our Pax-2 overexpression studies.

All nine Pax-2 isoforms retain exon 2 to 4 constituting the paired domain. The presence of single amino acid exchanges or a deletion of a 3-nucleotides in the paired domain in some of the Pax-2 proteins (Heller and Brändli, 1997) might, however, modify the DNA binding characteristics and target sequence specificities. EMSA could be performed to test the DNA binding activity of the nine Pax-2 isoforms. Extracts of cells transiently transfected with Pax-2 plasmids could be prepared and incubated with radioactively labeled DNA probes that contain putative Pax-2 binding sites. Binding reactions could then be analyzed by native polyacrylamide gel electrophoresis. The formation of DNA-protein complexes could be visualized by autoradiography. Finally, the specificity of DNA-protein complexes could be verified by competition and antibody supershift experiments.
Pax-2 isoforms diverge most significantly in their C-termini, which contain sequence motifs mediating transactivation (Dörfler and Busslinger, 1996; Lechner and Dressler, 1996). The transactivation potential of each splice variant could be assessed using transient transactivation assays. NIH 3T3 or 293 cells could be cotransfected with Pax-2 expression plasmids and a chloramphenicol acetyl transferase (CAT) reporter construct containing five tandem copies of a Pax-2 binding site upstream of a minimal promoter (Fickenscher et al., 1993). A β-galactosidase reporter plasmid could be used to monitor transfection efficiency. Standards methods would be employed to measure CAT activity in transfected cells (Gorman, 1985).

Overall, these experiments might allow to establish a correlation between the DNA binding activity and transactivation potential of individual Pax-2 isoforms and the phenotype observed in the overexpression experiments. Furthermore, the proposed *in vitro* experiments using Pax-2 isoforms generated by *in vivo* 'deletion mutagenesis' will complement previous structure-function studies carried out with *in vitro* mutagenized Pax-2 proteins (Dörfler and Busslinger, 1996; Lechner and Dressler, 1996).
7. References


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