Identification and Characterization of naturally occurring ecdysone response elements in Chironomus tentans and Drosophila melanogaster

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Identification and Characterization of Naturally Occurring Ecdysone Response Elements in *Chironomus tentans* and *Drosophila melanogaster*

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH for the degree of Doctor of Natural Sciences presented by Nadja Katharina Seibel

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1999
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ZUSAMMENFASSUNG


Zusammenfassung

SUMMARY

The steroid hormone ecdysone serves as a major signaling molecule in the life cycle of insects. One of the most complex developmental processes directed by this hormone is the metamorphosis from the larval to the adult form of the insect. The action of ecdysone is transduced by a heterodimeric receptor complex composed of two members of the nuclear receptor superfamily, the ecdysone receptor (EcR) protein and the Ultraspiracle (USP) protein. USP is the insect homolog of the vertebrate retinoid X receptor (RXR) and belongs to the so-called orphans, i.e., receptors with no known ligands. All nuclear receptors share a common structure with a highly conserved DNA-binding domain (DBD) and a less conserved ligand-binding domain (LBD). Upon ligand binding these receptors activate or repress transcription of a target gene through direct association with specific sequences known as hormone response elements (HREs) in the regulatory regions of these genes. While in vitro DNA binding studies of EcR/USP have revealed an extraordinary flexibility exceeding that for other members of the nuclear receptor superfamily, the small number of characterized natural ecdysone response elements (EcREs) belong primarily to a class exhibiting palindromic structures.

The isolation and characterization of natural EcREs from *D. melanogaster* and *C. tentans* is described in this work. A whole genome PCR approach was established to select for genomic EcR/USP binding sites using bacterially expressed proteins and a specific antibody against USP (AB11). Complexes formed by EcR/USP and AB11 bound to linker-modified genomic DNA fragments were selected by an immunoreaction and the DNA was amplified by PCR. After six consecutive cycles of selection and amplification, DNA fragments were cloned. Finally, four unique clones from *Drosophila* and six unique clones from *Chironomus*, respectively, were further analyzed by electrophoretic mobility shift assay (EMSA), sequencing, Southern hybridization, and in situ hybridization to isolated polytene chromosomes. They all represent as yet
unknown EcR/USP binding sites with the following interesting features. (i) Among all selected fragments, only two Drosophila clones harbor a known binding motif composed of AGGTCA half-sites arranged as direct repeats which can be read either as DR8 or as two interlocked DR1. A subfragment containing this motif reveals strong binding affinity to EcR/USP. Single half-sites of known EcREs are contained in all our fragments. Thus, also natural EcREs reflect a high level of structure variability. (ii) All fragments bind to in vitro translated EcR/USP as demonstrated by EMSA either indirectly (fragments as competitors) or directly (fragments as probes). (iii) In the presence of USP, the three Drosophila EcR (dEcR) isoforms, which have the same DBD, are equal in their binding affinity to the selected Drosophila fragments when tested in EMSA. On the other hand, binding of dEcR alone is isoform-specific and influenced by the ecdysone agonist muristerone A: homodimeric EcR binding to the fragments in the absence of hormone is changed into monomeric EcR binding in the presence of the ligand. (iv) Nearly all Drosophila fragments are able to bind to USP alone. This kind of binding is also visible with two Chironomus fragments but only in supershift experiments. As expected, the ligand has no influence on USP binding. (v) The majority of our selected fragments from both insect species can be correlated to puff-sites, as revealed by in situ hybridization experiments. For Chironomus, most of the fragments hybridize to puff-sites induced by heat shock. Whether these newly identified EcR/USP binding sites exhibit an activating or repressive function on the transcription of their target genes awaits further experimental investigation.
1. INTRODUCTION

In higher eukaryotes, hormones are involved in the control of a wide variety of biological processes, including development, homeostasis, cell differentiation, organ physiology and function, metabolism, reproduction, and metamorphosis. The hormone response is a consequence of the release from an endocrine gland of a ligand that circulates through the blood or hemolymph, and coordinately regulates responses in target tissues by acting through two different signaling pathways: signal transduction of the water-soluble peptide hormones which cannot pass the lipid cell membrane is mediated by receptor proteins located on the cell surface. Binding of the ligand causes conformational changes in the receptor which give rise to changed titers of second messengers (e.g. cAMP, Ca$^{2+}$, cGMP) in the target cell, which in turn control intracellular biochemical cascades. The action of the fat-soluble hormones which can penetrate their target cell, is mediated predominantly through intracellular proteins. Binding of the ligand to its receptor induces an allosteric change that allows the receptor-hormone complex to interact with cofactors and to bind to specific DNA sequences in the promoter region of the target gene (Evans, 1988; Beato, 1991). Ultimately, these receptors act as transcription factors mediating either activation or repression of the respective target gene in a ligand-dependent manner (Beato et al., 1995; Mangelsdorf et al., 1995). Although not all of the lipophilic hormones are either structurally or biosynthetically related, the existence of a common structure for their receptors supported the proposal that there is a large superfamily of genes whose products are ligand-responsive transcription factors (Evans, 1988; Green and Chambon, 1988; Beato, 1989; Carson-Jurica et al., 1990). A focus of current study is to understand the molecular basis of hormone-dependent transactivation mediated by members of the nuclear receptor superfamily which seems to be conserved from worms to insects to humans.
1.1 The nuclear receptor superfamily

Nuclear receptors represent a large family of zinc finger transcription factors that includes the ligand-inducible steroid, thyroid, vitamin D3 and retinoid acid receptors (Evans, 1988; Green and Chambon, 1988; Gronemeyer, 1991), as well as the so-called orphan receptors, which exhibit structures consistent with those of other superfamily members but lack a known ligand (Laudet et al., 1992; O'Malley and Conneely, 1992). The first hints about their common structural organization came from biochemical studies on steroid receptors (Wrange and Gustafsson, 1978; Carlstedt-Duke et al., 1982; Wrange et al., 1984; Carlstedt-Duke et al., 1988). These findings could be confirmed by comparison of the deduced amino acid sequences of the cloned cDNAs for virtually all members of the superfamily, beginning in the mid-1980s with cloning of the glucocorticoid receptor (GR) (Hollenberg et al., 1985; Miesfeld et al., 1986), the estrogen receptor (ER) (Green et al., 1986b), the thyroid receptor (TR) (Sap et al., 1986; Weinberger et al., 1986), the retinoic acid receptor (RAR) (Giguère et al., 1987; Petkovich et al., 1987), the retinoid X receptor (RXR) (Mangelsdorf et al., 1990), and the first orphan receptors (Giguère et al., 1988). As depicted in Figure 1, all nuclear hormone receptors are composed of functional domains (A-F) that mediate DNA binding, dimerization, ligand binding, formation of the heat shock protein complex, and transcriptional activation (Krust et al., 1986; Kumar et al., 1987). As a consequence of their modular structure, each domain is able to act in a relatively independent fashion (Evans, 1988).
Introduction

A.

\[
\text{NH}_2 \quad \begin{array}{c}
\text{A/B} \quad \text{C} \quad \text{D} \quad \text{E} \quad \text{F} \quad \text{COOH}
\end{array}
\]

\[
\begin{array}{c}
\text{C} \quad \text{C} \quad \text{Zn} \quad \text{C} \quad \text{C} \\
\text{C} \quad \text{P-box} \quad \text{Zn} \quad \text{C} \\
\text{C} \quad \text{D-box} \quad \text{C} \quad \text{C}
\end{array}
\]

B. Domain Function(s)

<table>
<thead>
<tr>
<th>Domain</th>
<th>Function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/B</td>
<td>Transactivation (AF-1)</td>
</tr>
</tbody>
</table>
| C      | DNA binding (DBD) 
Dimerization interface |
| D      | Nuclear localization 
Corepressor binding |
| E      | Ligand binding (LBD) 
Transactivation (AF-2) 
Dimerization interface 
Nuclear localization 
Coactivator binding 
Binding of HSP90 |
| F      | Unknown |

Figure 1. The structural and functional characteristics of nuclear hormone receptors. (A) The modular structure of nuclear hormone receptors with the five distinct domains (A-F). The two regions showing the highest degree in conservation among all family members, namely the C domain and E domain, are indicated with the black boxes. The C domain which is responsible for DNA binding is shown in more detail (C, cysteine residue; Zn, zinc atom). (B) The five domains can be matched to defined functions. For further explanation see text.
The most highly conserved domain (50-90 %) is the C domain, also called DNA-binding domain (DBD) (Evans, 1988). The whole domain comprises approximately 80 aa residues and is rich in arginine, lysine, and cysteine residues (Hollenberg et al., 1985; Weinberger et al., 1985). X-ray crystal structure and nuclear magnetic resonance (NMR) analyses of several DBDs (Rastinejad et al., 1995; Luisi et al., 1991; Katahira et al., 1992; Lee et al., 1993; Schwabe et al., 1993) demonstrated that the first 66 aa residues with nine conserved cysteines contain the two zinc finger motifs, each of them with a zinc atom tetrahedrally coordinated to four cysteines (Fig. 1A). This core region, which is conserved across all members of the family (Klug and Rhodes, 1987; Severne et al., 1988; Evans, 1988) forms two α-helices. The N-terminal zinc finger (CI) is rich in hydrophilic aa and is more highly conserved than the more C-terminal zinc finger (CII) which is highly basic and rich in lysine and arginine. Direct contact with the major groove of the DNA half-site of the corresponding hormone response element is mediated by the 3 aa of the P-box, which is located at the C-terminal end of CI (Fig. 1A). Altering the amino acids in the P-box can alter the half-site specificity of the receptor (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evans, 1989). The D-box which is located between the first cysteines of CII (Fig. 1A) is not only important for the recognition of the spacing between the two DNA half-sites (Umesono and Evans, 1989), but is also involved in dimerization of two GR-DBDs (Dahlman-Wright et al., 1991). Another dimerization interface, the T-box, which is just C-terminal of the core region (Fig. 1A), was reported to be necessary for heterodimerization activity of the vitamin D3 receptor (VDR) (Hsieh et al., 1995; Quack et al., 1998) and homodimerization of RXR (Rastinejad et al., 1995). An additional α-helix located at the C-terminal extreme of the DBD, termed the A-box (Fig. 1A), makes minor groove contact with bases at the 5' end of the DNA half-site (Wilson et al., 1992; Kurokawa et al., 1993).

In comparison with the DBD, the E domain or ligand-binding domain (LBD), which is located at the C-terminal part of nuclear receptors, is less conserved (15-60 %). This region of about 250 aa with a strong hydrophobic character (Green et al., 1986a)
performs a number of functions that include ligand binding, dimerization, transcriptional activation and repression (Kumar and Chambon, 1988; Hollenberg and Evans, 1988; Beato et al., 1995; Mangelsdorf et al., 1995). In addition, the LBD is involved in nuclear localization (Picard and Yamamoto, 1987) and heat shock protein binding (Pratt, 1992). Crystallographic data and structure functional analysis revealed that LBDs primarily fold as antiparallel $\alpha$-helical sandwiches composed of eleven or twelve $\alpha$-helices (H1 to H12) (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995). An exception to the rule is the LBD of the peroxisome proliferator-activated receptor (PPAR) which contains thirteen $\alpha$-helices (Nolte et al., 1998). Upon ligand binding, several major changes are induced within the LBD which allows the recruitment of coactivator proteins. This leads to transcriptional activation of the target gene mediated through the ligand-dependent activation function 2 (AF-2) in H12 (Horwitz et al., 1996; Glass et al., 1997).

The remaining two domains at each end and the one in the middle are not very much conserved among the members of the nuclear receptor family. The N-terminal A/B domain is highly variable in length (5-603 aa) and structure. It harbors a ligand-independent transcriptional activation function 1 (AF-1) (Hollenberg and Evans, 1988; Bocqucl et al., 1989; Tora et al., 1989) which can interact with the basal transcription machinery (McEwan et al., 1993; Rochette-Egly et al., 1997) or with members of the p160 coactivator family (Webb et al., 1998). The role of the D domain (45 aa) located between the DBD and LBD is mainly nuclear localization (Picard and Yamamoto, 1987) and association with corepressors mediated through the CoR-box (Chen and Evans, 1995; Hörlein et al., 1995). This region also serves as flexible hinge allowing rotational differences between the other domains. The function of the F domain at the C-terminal end of nuclear receptors is much undefined. However, recent studies could show a repressive influence of this domain on AF-2 activity caused by masking parts of the LBD (Hadzopoulou-Cladaras et al., 1997). Furthermore, the F domain can affect the different responses of ER to agonists and antagonists (Montano et al., 1995; Nichols et al., 1998). Some receptors are totally lacking the F domain.
Introduction

1.2 Monomers, homo- and heterodimers

Members of the nuclear receptor superfamily function primarily as dimers (Evans, 1988; Forman and Samuels, 1990). As described above, there are two independent dimerization functions present in nuclear receptors, a weak one located within the DBD (Kurokawa et al., 1993; Perlmann et al., 1993; Gronemeyer and Moras, 1995) and a strong one located in the LBD (Kumar and Chambon, 1988; Zhang et al., 1994). Although there exists no dimerization via DBDs in solution, this interface is involved in highly cooperative binding of two monomers on the appropriate target DNA as shown for RXR, TR, RAR or VDR (Perlmann et al., 1993; Zechel et al., 1994a; Zechel et al., 1994b; Mader et al., 1993a; Towers et al., 1993). Dimerization via the LBD is mainly mediated by α-helices H9 and H10 and to a lesser extend by H8 and H11 which was demonstrated for LBDs of human RXRα, RARγ, TRα and ERα (Bourguet et al., 1995; Brzozowski et al., 1997; Renaud et al., 1995; Wagner et al., 1995).

According to their oligomerization status, nuclear receptors can be categorized into different subfamilies. Usually, steroid hormone receptors, which mainly form homodimers, are distinguished from a large diverse subfamily of receptors for non-steroid ligands, such as thyroid hormone (TR), retinoids (RAR and RXR), and eicosanoids (PPAR), as well as many orphan receptors. Unlike steroid receptors, most of these receptors function as heterodimers with RXR and thus represent highly dynamic transcription factor complexes due to the association of two receptor subunits with distinct structural and functional features (Leid et al., 1992; Mangelsdorf and Evans, 1995). Due to this potential, RXR is seen as a 'master regulator' for many different signaling pathways. In heterodimeric complexes with TR and VDR, RXR is suggested to be a silent partner which is not able to bind its ligand (Kurokawa et al., 1994; Forman et al., 1995b). However, RXR is known to play an active role together with PPAR (Kliwer et al., 1992b), liver X receptor (LXR) (Willy et al., 1995), farnesoid X receptor (FXR) (Forman et al., 1995a), and nerve growth factor-induced receptor B (NGFI-B) (Perlmann and Jansson, 1995; Forman et al., 1995b). In the case
of the RXR/RAR heterodimer, RXR is only able to bind ligand after the addition of an RAR ligand (Forman et al., 1995b; Schulman et al., 1997). Depending on the DNA element, RXR can also form homodimers that bind and are stabilized by 9-cis retinoic acid (9-cRA) (Zhang et al., 1992; Lehmann et al., 1993).

The major group of the nuclear receptor family, the orphans, may not only form heterodimers with RXR (see above) but also homodimers as shown for the germ cell nuclear factor (GCNF) (Chen et al., 1994; Yan et al., 1997) and the hepatocyte nuclear receptor 4 (HNF-4) (Jiang et al., 1995). Other orphan receptors bind their cognate DNA response element only as monomers, e.g., the retinoid orphan receptor α (RORα) (Winrow et al., 1998) or the steroidogenic factor 1 (SF-1) (Wilson et al., 1993). In addition, receptors like the chicken ovalbumin upstream promoter transcription factors (COUP-TFs) or NGFI-B act as monomers, homodimers or heterodimers together with RXR (Wilson et al., 1991; Cooney et al., 1992; Kliewer et al., 1992a; Mangelsdorf and Evans, 1995; Drouin et al., 1998).

1.3 Hormone response elements

Interaction of nuclear receptors with specific DNA sequences known as hormone response elements (HREs) is a prerequisite for the regulation of their target gene (Evans, 1988; Beato, 1989). These HREs function in a position- and orientation-independent fashion and thus behave like ligand-dependent transcriptional enhancers or, in some cases, silencers (Benoist and Chambon, 1981; Banerji et al., 1983; Chandler et al., 1983; Saatcioglu et al., 1994). Since the identification of the first response element for GR (GRE) in the mouse mammary tumor virus (MMTV) (Payvar et al., 1983), comparison with other elements from various receptors revealed strong structural homologies among different HREs. Response elements for dimeric complexes comprise approximately 15 bp containing two half-sites, arranged in either a palindromic order (PALs) or as direct repeats (DRs) (Beato, 1989; Glass, 1994). Evolutionary studies showed that such composed elements derived from a duplication of PuGGTCA where
Pu means a purine, i.e. A or T (Umesono and Evans, 1989; Martinez et al., 1991; Gronemeyer and Laudet, 1995).

Recognition and tight binding to the respective element is determined by the dimerization properties of the receptor as well as the amino acid sequence of the DBD (Kumar et al., 1987). For steroid hormone receptors which form homodimers, the HREs are predominantly composed as palindromes. Due to variations of the 3 amino acid residues in the P-box, the steroid receptors are subdivided in two classes, the GR subfamily targeting the half-site AGAACA and the ER subfamily which targets the half-site AGGTCA (Tsai et al., 1988; Umesono and Evans, 1989; Zilliacus et al., 1994). The most potent response elements, the DRs composed of a core AGGTCA half-site, are recognized by members of the retinoid/thyroid receptor family which form heterodimers with RXR (Leid et al., 1992; Stunnenberg, 1993; Glass, 1994). Additionally, they are targets for homo- and heterodimeric orphan receptors, whereas monomeric orphan receptors bind to single extended half-sites (Wilson et al., 1993; Mangelsdorf and Evans, 1995).

Further specificity of an HRE is given by the composition of the 5' flanking region (Vivanco Ruiz et al., 1991; Mader et al., 1993b) and the spacing between each half-site. The latter is manifested in the so-called 1-to-5 rule describing homo- and heterodimeric binding of VDR, TR, RAR and RXR to direct repeated half-sites spaced by 1, 2, 3, 4, and 5 nucleotides (DR1, DR2, DR3, DR4, DR5) (Umesono et al., 1991; Heery et al., 1994; Mangelsdorf and Evans, 1995). Detailed studies on RXR complexes could demonstrate that the different spacing is responsible for the polarity of the bound dimer: on DR2, DR3, DR4, and DR5, RXR occupies the 5' half-site and its partner (e.g. VDR, TR, RAR) is located on the 3' half-site of the motif (Kurokawa et al., 1993; Perlmann et al., 1993; Zechel et al., 1994b). On the contrary, a reversed orientation is detected for RAR/RXR heterodimers bound to DR1 (Kurokawa et al., 1994). Interestingly, this element is also recognized by RXR homodimers (Zechel et al., 1994b).
Introduction

1.4 Transactivation and transrepression

1.4.1 The role of ligand

The final aim of nuclear hormone receptors is transcriptional regulation of their target genes, which is achieved through two autonomous activation functions (AFs), a constitutive AF-1 in the N-terminal A/B domain and a ligand-induced AF-2 located at the C-terminal end of the LBD (Danielian et al., 1992; Nagpal et al., 1993; Barettino et al., 1994; Durand et al., 1994; Metzger et al., 1995). Both AFs act in a promoter- and cell-specific fashion (Bocquel et al., 1989; Tora et al., 1989) and interact with one another to perform full transactivation (Nagpal et al., 1993; Kraus et al., 1995).

Prior to transcriptional activation of the target gene, the receptors themselves have to be transformed from a repressive to an activated status which is different for steroid and non-steroid receptors, often designated as class I and class II receptors, respectively. Unliganded class I receptors like GR are associated via their LBD with a large multiprotein complex including heat shock proteins (HSPs), most particularly HSP90 (Pratt and Toft, 1997). Whereas the inactive progesterone receptor (PR) and ER seem to be nuclear (Press and Greene, 1988; Puca et al., 1986), inactive receptor-HSP complexes of GR, the mineralocorticoid receptor (MR), and probably the androgen receptor (AR) are thought to be located in the cytoplasm (Sackey et al., 1996; Picard and Yamamoto, 1987). In the presence of their ligand, the HSPs dissociate and the hormone-bound receptors are now capable of dimerization, of translocation to the nucleus, of binding to DNA and of transactivation. In contrast, receptors belonging to class II, i.e. TR, RAR, RXR, VDR, PPAR, and the orphans are not inactivated by binding to HSPs. Even in the absence of hormone they are associated as dimers or monomers with their cognate HRE. However, the unliganded receptors form complexes with corepressors such as the nuclear receptor corepressor (N-CoR) (Hörlein et al., 1995; Heinzel et al., 1997) or the silencing mediator of retinoid and thyroid receptors (SMRT) (Chen and Evans, 1995). Hormone binding causes conformational changes in the receptor which results in the release of corepressors and turning back of the AF-2.
helix towards the hormone-binding pocket (Renaud et al., 1995; Wagner et al., 1995). The subsequent interaction with various coactivators, e.g. members of the steroid receptor coactivator 1 (SRC-1) family (Glass et al., 1997; Voegel et al., 1998; Yuan et al., 1998), p300/CREB-binding protein (CBP) (Goldman et al., 1997) and p300/CBP-associated factor (PCAF) (Blanco et al., 1998) or directly with the basal transcription machinery through AF-1 and AF-2 are common to all nuclear receptors. In agreement with the structural conservation of the coactivator interaction surface in the receptors (Feng et al., 1998; Nolte et al., 1998), most AF-2 domain-binding proteins contain short conserved LXXLL interaction motifs, where L means leucine and X any amino acid residue (Heery et al., 1997; Torchia et al., 1997).

In addition to the above described mechanisms for transcriptional repression, there are several other ways of gene silencing, either on the level of DNA or on the level of proteins. For receptors like GR, VDR or TR the existence of so-called negative response elements has been reported, which are repressed in the presence of ligand and are only activated by unliganded receptors (Demay et al., 1992; Drouin et al., 1993; Carr and Wong, 1994). Possible explanations for this ligand-induced repression might be (i) the location of the HRE downstream of the TATA-box (Chatterjee et al., 1989; Carr et al., 1992), (ii) the DNA sequence itself acting as an allosteric effector on the receptor (Lipkin et al., 1992; Towers and Freedman, 1998) or (iii) the spacing between the two half-sites (Kurokawa et al., 1995). Negative regulation due to protein-protein interactions is mediated through direct competition for DNA-binding sites (Cooney et al., 1992; Kliewer et al., 1992a; Tran et al., 1992; Segars et al., 1993) or sequestering of receptors, like the formation of RXR homodimers instead of thyroid hormone (T3) responsive TR/RXR heterodimers (Lehmann et al., 1993). In addition, response to T3 can be repressed by the formation of PPAR/RXR (Juge-Aubry et al., 1995) or VDR/RXR (Yen et al., 1996) heterodimers instead of TR/RXR heterodimers (dominant-negative effects).
1.4.2 The role of chromatin

It has been shown that the chromatin structure plays an important role in gene activation (Adams and Workman, 1993; Pazin et al., 1994; Venter et al., 1994; Wolffe, 1994). Normally, inactive chromatin is packed into nucleosomes, which prevents binding of transcription factors. However, as demonstrated in recent studies (Wolffe et al., 1997; Fondell et al., 1999), parts of sites have to be properly oriented and accessible for binding of nuclear receptors as this is absolutely needed for subsequent ligand-induced changes of the chromatin structure. The transformation into active chromatin is provided by the liganded nuclear receptors bound to coactivators (see above) which contain histone acetyltransferase (HAT) activity and which make contact to the basal transcription machinery. In repressed chromatin, the histones are deacetylated through corepressors bound to the non-liganded receptors. It turned out that remodeling of chromatin rather than total disruption of positioned nucleosomes is the favored mechanism responsible for this transformation (Archer et al., 1991; Schild et al., 1993; Truss et al., 1995; Scholz et al., 1999).

1.5 Hormonal influences on insect development

The postembryonic development of holometabolous insects is strongly regulated by two different classes of hormones, the ecdysteroids and the juvenile hormones (JH) (reviewed by Riddiford, 1993). Beginning from the hatched egg over several larval instars and the puparium to the final stage of the highly motile and reproductively active imago, each molt is preceded by peaks of ecdysone (Fig. 2A). Whether a larval molt or metamorphosis will take place is determined by the presence or absence of JH at the time of ecdysone release (Riddiford, 1978; Berger et al., 1992). Ecdysone, which is a steroid hormone, is secreted by the prothoracic gland as a pro-hormone (Bollenbacher et al., 1976) and is converted in the fat body and some other organs into the physiologically active form 20-hydroxyecdysone (referred to here as ecdysone) (Hodgetts et al., 1977). The ecdysone pulses during the first larval instars trigger
molting of the cuticle. This apparently tissue-specific response contrasts with the pleiotropic effects of ecdysone during the last larval instar, when one or more successive hormone pulses lead to a complex series of behavioral and developmental changes that culminate in puparium formation and the onset of terminal differentiation into the adult fly (Richards, 1981). The first of these successive pulses at the end of larval development induces destruction of a subset of larval tissues and morphogenesis of the imaginal discs to form rudiments of the adult cuticle (Robertson, 1936). A second pulse 10-12 h after puparium formation, which marks prepupal-to-pupal transition triggers adult head eversion, histolysis of the remaining larval tissues and the onset of adult tissue differentiation.

The polytene chromosomes of the salivary glands of higher Diptera have proven to be a unique tool to study the hormonal control of development. In these chromosomes, many genes are directly visible as large puffs when active. Puffs appear and regress in a precise temporal sequence. Clever and Karlson (1960) showed that injection of ecdysone into late third instar larvae of the midge *Chironomus tentans* caused a rapid appearance of two new puffs (I-18C and IV-2B), whose induction was not prevented by the addition of protein synthesis inhibitors (Clever, 1964). This was the first demonstration in any organism that a hormone could affect gene activity. Similar results were obtained with experiments on *Drosophila melanogaster* polytene chromosomes performed by Ashburner (Ashburner et al., 1974). Here, six early puffs appeared within 15-30 min of exposure to ecdysone followed by the appearance of more than hundred puffs several hours later. As in *C. tentans*, induction of early puffs was observed even in the presence of protein synthesis inhibitors, whereas their regression was abolished under these conditions. For the induction of the late puffs protein synthesis was absolutely required. Based on these observations Ashburner proposed a model for the molecular mechanisms during the onset of metamorphosis where ecdysone directly activates a small set of early genes encoding transcription factors which subsequently induce transcription of the late genes. Thus, the inhibitory effect of ecdysone on the late
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genes is only reversed in the presence of early gene products. Repression of the early genes is regulated by their own expression via a negative feedback mechanism.

Up to now, four of the early puff genes in *D. melanogaster* have been defined at the molecular level. Three of them are induced by both the late larval and the prepupal ecdysone pulses and are expressed in many target tissues. The first, which maps to the cytogenetic locus 2B5 harbors the gene for the Broad-Complex (BR-C), which encodes a family of C2H2 zinc finger proteins (DiBello et al., 1991). BR-C consists of a large number of overlapping transcription units exhibiting various functions. During the mid-third instar it is required for induction of the glue genes (Crowley et al., 1984; Hansson and Lambertsson, 1989; Georgel et al., 1991; Guay and Guild, 1991), whereas it is needed for normal puffing at the end of larval development. During prepupal development it regulates normal imaginal disc morphogenesis (Kiss et al., 1988), complete metamorphosis of salivary glands, gut, and dorsal flight muscles (Restifo and White, 1992), as well as the remodeling of the CNS (Restifo and White, 1991). The second early gene localized to the puff 74EF encodes two transcription factors, designated E74A and E74B (Burtis et al., 1990). They both share a common 3’ end and contain a so-called ETS-domain, which is characteristic for certain proto-oncogenes. Transcription of E74B in response to an ecdysone pulse always precedes that of E74A (Karim and Thummel, 1991). The third analyzed early gene localizes to the 75B puff and consists of three overlapping transcription units, *E75A*, *E75B*, and *E75C* (Feigl et al., 1989; Segraves and Hogness, 1990) which code for members of the nuclear receptor superfamily. *E75B* is unique in that it contains only one zinc finger. In addition to the function of *E75B* during metamorphosis (see below), it has been suggested that *E75* is involved in embryonic gut morphogenesis (Bilder and Scott, 1995). Another early gene in *Drosophila*, *E93*, shows a strictly stage- and tissue-specific expression pattern (Baehrecke and Thummel, 1995). In the salivary glands, CNS and in the imaginal discs *E93* is only expressed in late prepupae, only induced by the prepupal ecdysone pulse (Fig. 2B). It encodes a transcription factor that is involved in the histolysis of the salivary glands (see below).
Figure 2. Ecdysone-regulated development of *Drosophila melanogaster*. (A) Each of the six developmental stages (embryo, three larval instars, prepupa, pupa, adult) is characterized by changes in the ecdysone titer. The X-axis shows the time-scale in days, the Y-axis shows the 20-hydroxyecdysone equivalents from whole body homogenates. (B) The ecdysone-induced events at the onset of metamorphosis are mediated by cross-regulation among members of the nuclear receptor superfamily. The induction of early and late genes is triggered through the rise in ecdysone titer both at the end of third larval instar and in late prepupa. Induction of βFTZ-F1 through DHR3 is repressed by heterodimerization between DHR3 and E75B. Induction is depicted by green arrows, repression by red arrows. For further explanation see text.
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1.6 Nuclear receptors in insects

Molecular studies of the ecdysone regulatory hierarchies were greatly facilitated by isolation of genes encoding members of the nuclear receptor superfamily, starting in the late 1980s. Functional analyses in *Drosophila* revealed that most of these nuclear receptors are expressed at the onset of metamorphosis. Furthermore, they provided evidence that there are cross-regulatory interactions among ecdysone-induced transcription factors (reviewed by Thummel, 1996). The proposed model of multistep regulatory hierarchies, which is in contrast to the two-step hierarchy of Ashburner, is shown schematically in Figure 2B. To date, sixteen members of the nuclear receptor superfamily have been identified in *Drosophila*, several of them are also known in other insect species. With the exception of one, they all represent orphan receptors.

1.6.1 Functional receptors during embryogenesis

Three of the receptor genes isolated in *Drosophila* encode highly divergent members of the superfamily in that they lack the LBD. Two of them, *knirps* (*kni*) (Nauber et al., 1988) and *embryonic gonad* (*egon*) (Rothe et al., 1989), are expressed exclusively during embryogenesis, whereas the embryonic expression of *knirps-related* (*knrl*) (Oro et al., 1988; Rothe et al., 1989) continues in larvae and adults.

The nuclear receptor encoded by the *Drosophila* gene *tailless* (*til*) (Pignoni et al., 1990) is unique because of its amino acid composition in the P-box and a D-box containing seven amino acid residues. The zygotically active *til* is involved in the formation of the posterior and anterior termini of the embryo.

The function of the *Drosophila* homolog of the vertebrate HNF-4 (dHNF-4) (Zhong et al., 1993) is not fully elucidated. Expression of *dHNF-4* is restricted to embryonic tissues like midgut, Malpighian tubules and the fat body.
1.6.2 Receptors with functions in metamorphosis

Of the eleven *Drosophila* nuclear receptors which are known to function during metamorphosis, a direct regulation by ecdysone could be shown for nine family members, i.e. E75 (Segraves and Hogness, 1990), DHR3 (Koelle et al., 1992), DHR38 (Sutherland et al., 1995), DHR39 (Ohno and Petkovich, 1992; Ayer et al., 1993), DHR78 and DHR96 (Fisk and Thummel, 1995), βFTZ-F1 (Lavorgna et al., 1991), E78 (Stone and Thummel, 1993), and the ecdysone receptor (EcR) (Koelle et al., 1991). Interestingly, EcR is the only ligand-dependent nuclear receptor identified to date. Together with the gene product of *ultraspiracle (usp)* (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990) it forms a complex which is able to transactivate a target gene (see next chapter). Precise timing of expression is regulated by receptor interactions and variation of the ecdysone titer (Thummel, 1995) (Fig. 2B).

Expression of *DHR78* starts at the mid-third larval instar, when the ecdysone concentration is low. It binds as a homodimer to a variety of DR elements, with preference for DR1 (Fisk and Thummel, 1995). On some response elements the DHR78 homodimer can act as a negative regulator of ecdysone-mediated transactivation due to competition for DNA-binding sites (Zelhof et al., 1995b). Its critical role in regulating the onset of metamorphosis was demonstrated by mutations in *DHR78* which result in a blockage of ecdysone-regulated gene expression in mid-third instar larvae (Zelhof et al., 1995b; Fisk and Thummel, 1998). Based on these findings and the fact that the expression of *DHR78* takes place when the ecdysone titer is low, it has been suggested that *DHR78* is maybe regulated by an as yet unknown hormone which would trigger the events in the mid-third larval instar (Fisk and Thummel, 1998). However, evidence for this hypothesis remains elusive.

The rise in ecdysone titer at the end of the third larval instar leads to the expression of the early genes and to a slightly delayed expression of *E78B*. This so-called early-late gene is induced by ecdysone – like the early genes – but it also requires ecdysone-induced protein synthesis for maximal transcription – like the late genes (Stone and
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Thummel, 1993). It has been suggested that the early gene product E74A is involved in
the highly stage-specific induction of E78B (Fletcher and Thummel, 1995). Three other
early-late genes encoding DHR39, DHR3 and E75B are also induced by the late larval
ecdysone pulse. While E75B cannot bind to DNA because it lacks the first zinc finger
(Sehraves and Hogness, 1990), DHR3 which has its peak expression in the early
prepupa (Huet et al., 1995) can bind to a single AGGTCA core sequence preceded by an
AT-rich sequence (Horner et al., 1995). DHR3 directs the larval-to-prepupal transition
and exhibits both repressive and activating functions: the repressive effect of DHR3 on
the expression of the early genes is probably mediated by direct interaction with EcR,
which has been shown in a yeast two-hybrid assay (White et al., 1997). Together with
E75B, DHR3 directs the appropriate induction of the mid-prepupal gene βFTZ-F1
(Thummel, 1997). The short period of low ecdyson alone and two high affinity binding
sites for DHR3 in the promoter of βFTZ-F1 seem to be sufficient for the expression of
βFTZ-F1, which is determined by negative autoregulation (Woodard et al., 1994; Lam
et al., 1997). The exact timing of this induction correlates with a decrease in E75B
expression. E75B can form heterodimers with DHR3 which bind to sites in the βFTZ-
F1 promoter but fail to transactivate (Sehraves and Hogness, 1990; White et al., 1997).
Thus, direct interaction of E75B with DHR3 inhibits the induction of βFTZ-F1.

The prepupal-to-pupal transition in response to a second ecdyson pulse leads to re-
induction of the early genes and expression of E93, a stage-specific early gene which
precedes the onset of salivary gland histolysis (Baehrecke and Thummel, 1995). The
competence for the stage- and tissue-specific expression pattern of E93 is provided by
βFTZ-F1, which has been demonstrated by mutational analyses (Woodard et al., 1994;
Broadus et al., 1999).

A possible role of DHR96, which is transcribed at the onset of metamorphosis still
remains uncharacterized. Due to its unique P-box it preferentially binds to a response
element in the promoter of the ecdyson-inducible Drosophila heat shock gene hsp27
(Fisk and Thummel, 1995).
The expression pattern of two other Drosophila receptors encoded by seven-up (svp) (Mlodzik et al., 1990) and DHR38 (Sutherland et al., 1995) is strictly tissue-specific. The two isoforms of SVP, which are homologous to the vertebrate COUP-TFs, are expressed in the photoreceptor precursor cells and are needed for adult eye development. SVP binds to response elements arranged as direct repeats of the half-site AGGTCA, with best binding to DR1 (Zelhof et al., 1995a). Furthermore, it is involved in negative regulation of ecdysone signaling via direct binding to EcR or competition for DNA-binding sites (Zelhof et al., 1995a). The orphan receptor DHR38 is transcribed at the onset of metamorphosis and is required for adult cuticle development (Kozlova et al., 1998). Like its vertebrate homolog NGFI-B it can bind to a single AGGTCA half-site as a monomer. Recently, several groups reported a direct interaction of DHR38 with USP (Sutherland et al., 1995; Crispi et al., 1998).

1.7 The ecdysone receptor complex

Evidence for the existence of an ecdysone receptor initially consisted in the detection of a protein in extracts of cultured cells that exhibited specific binding to a 23 bp ecdysone responsive element in the promoter of the ecdysone-inducible Drosophila heat shock gene hsp27 (Riddihough and Pelham, 1987). The question as to whether this protein also exhibited ecdysone-specific binding, however, remained open until cloning of the EcR gene (Koelle et al., 1991). Although EcR was shown to restore hormone responsiveness in ecdysone-resistant Drosophila SL-2 cells (Koelle et al., 1991), transfection of EcR failed to confer ecdysone responsiveness to mammalian CV1 cells, indicating that some necessary auxiliary factor was lacking in these heterologous cells. Several groups have found that heterodimerization of EcR with the insect homolog of RXRα, USP, is required for effective binding of ecdysone, sequence-specific interaction with DNA and transcriptional activation of target genes (Koelle, 1992; Yao et al., 1992; Yao et al., 1993; Thomas et al., 1993). Indeed, EcR and USP co-localize on ecdysone responsive loci of Drosophila polytene chromosomes (Yao et al., 1993). In contrast to the heterodimers composed of EcR/SVP, EcR/DHR3, and USP/DHR38 (for references
see above), the ecdysone receptor complex (EcR/USP complex) is the only ecdysone-inducible heterodimer known to date.

In *Drosophila*, *EcR* encodes three isoforms, EcR-A, EcR-B1, and EcR-B2 which only differ in their N-terminal A/B domain (Talbot et al., 1993). EcR is widely expressed throughout insect development with the A isoform predominantly expressed in developing adult structures and tissues and the B1 isoform primarily expressed in larval cells that are fated to die (Talbot et al., 1993). The expression pattern of EcR-B2 has not yet been determined because no antibody is available that would recognize the 17 amino acid EcR-B2 specific region. All three EcR isoforms acquire the ability to bind to DNA upon heterodimerization with USP (Koelle, 1992; Talbot et al., 1993). The proposal that different combinations of EcR isoforms with USP are required for different tissue responses to ecdysone could be confirmed recently by mutational analysis (Bender et al., 1997).

In contrast to the three isoforms encoded by *EcR*, only one form of USP is known in *Drosophila*. USP is widely expressed, both spatially and temporally with highest level in adults and embryos and lower level in larvae (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990; Henrich et al., 1994). Like its vertebrate homolog, USP is able to bind as a homodimer to DR elements composed of AGGTCA half-sites with different spacing (D'Avino et al., 1995; Antoniewski et al., 1996; Vögtl et al., 1998). A possible ligand, if there is any, has not yet been found. However, a possible role in JH signaling has been proposed from experiments with *usp* mutants. In mutant animals, a supernumerary cuticle is formed instead of the normally produced pupal cuticle at the time of pupariation (Hall and Thummel, 1998). In addition, a recent study has proposed that USP is a JH receptor, although this binding is not saturable and of low affinity (Jones and Sharp, 1997). Clear evidence for this is still lacking and an effect of JH on the transactivation function of USP has not yet been demonstrated.
Homologs of *Drosophila* EcR and USP have been isolated from several insect species including *Chironomus tentans*, which, besides *Drosophila*, was used for our investigations (see below). On the level of amino acid sequence the *Chironomus* EcR (cEcR) exhibits 95% identity in the DBD and 75% identity in the LBD compared to its *Drosophila* homolog (Imhof et al., 1993). However, no EcR isoforms are known in *Chironomus*, which is in contrast to the *Chironomus* USP (cUSP) (Vögltli et al., 1999). In comparison to the *Drosophila* homolog, the deduced amino acid sequences of *Chironomus* USP-1 and USP-2 show 95% identity in the DBD (Vögltli et al., 1999). As demonstrated in *Drosophila*, cEcR and cUSP co-localize on ecdysone responsive loci of *Chironomus* salivary gland chromosomes (Wegmann, 1994).

### 1.8 Ecdysone response elements

Transcriptional regulation by the EcR/USP complex is mediated through binding to specific sequences, the so-called ecdysone response elements (EcREs), in the vicinity of ecdysone responsive target genes. Although several genes are known that are directly regulated by ecdysone, high-resolution mapping of the corresponding EcREs has been achieved only for a small set of tissue- and stage-specific genes from *Drosophila*. Similar to the HREs of vertebrate steroid hormones, most of the characterized EcREs are composed as imperfect palindromes with two hexameric half-sites spaced by one nucleotide. However, high affinity binding of the EcR/USP complex is also detected on direct repeats.

The first characterized EcRE was mapped to the promoter of the *Drosophila* heat shock gene *hsp27*, a gene which is activated by both heat shock and ecdysone and which is expressed in prepupae and in ovarian nurse cells (Riddihough and Pelham, 1986). The *hsp27* EcRE is located in a 23 bp region upstream of the start codon (Riddihough and Pelham, 1987) (for sequence see Table 1). It is the most efficient response element of genomic origin identified to date.
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Table 1. Comparison of natural EcREs from *Drosophila melanogaster*

<table>
<thead>
<tr>
<th>EcRE</th>
<th>Position</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hsp27</em> EcRE</td>
<td>-545/-533</td>
<td>GGTCAATGCACT</td>
</tr>
<tr>
<td><em>Eip28/29</em> upstream element</td>
<td>-447/-435</td>
<td>AGGTTAATGACCA</td>
</tr>
<tr>
<td><em>Eip28/29</em> proximal element</td>
<td>+2674/+2662</td>
<td>AGGTCACTACTTT</td>
</tr>
<tr>
<td><em>Eip28/29</em> distal element</td>
<td>+4452/+4440</td>
<td>AGTCACTGGGGTCGTA</td>
</tr>
<tr>
<td><em>Fbp1</em> EcRE</td>
<td>-91/-103</td>
<td>GGGTGAGTGAATT</td>
</tr>
<tr>
<td><em>Lsp-2</em> EcRE</td>
<td>-75/-63</td>
<td>CGGTCAATGGCG</td>
</tr>
<tr>
<td><em>Sgs-3</em> proximal element</td>
<td>-50/-38</td>
<td>GGTCAATGCAAG</td>
</tr>
<tr>
<td><em>Sgs-3</em> element I</td>
<td>-406/-394</td>
<td>GGGTCAATAGCCG</td>
</tr>
<tr>
<td><em>Sgs-3</em> element II</td>
<td>-753/-741</td>
<td>CTTGAGTGAATG</td>
</tr>
<tr>
<td><em>Sgs-4</em> element I</td>
<td>-308/-296</td>
<td>AGTTGAGGCG</td>
</tr>
<tr>
<td><em>Sgs-4</em> element II</td>
<td>-405/-393</td>
<td>GGTAAAGTAAACT</td>
</tr>
<tr>
<td><em>Sgs-8</em> element</td>
<td>-66/-78</td>
<td>ATTTGAGGCG</td>
</tr>
</tbody>
</table>

The nucleotide sequences of the palindromic EcR/USP binding sites from seven *Drosophila* genes and their position relative to the transcription initiation site are shown. The hexameric half-sites are depicted as bold letters. Note that the *Eip28/29* distal element contains a degenerated PAL1 as well as a DR3 (nucleotides that contribute to the half-sites of DR3 are underlined).

In the *cis*-regulatory sequences of the *ecdysone inducible protein 28/29* (*Eip28/29*) three EcR/USP binding sites have been identified (Cherbas et al., 1991). The upstream element shows only a weak induction through ecdysone when tested in transfection assays, maybe due to masking of the sequence by proteins other than EcR/USP (Cherbas et al., 1991). The other two regions, the proximal and distal element, are located downstream of the start codon. All three elements contain a degenerated PAL1 (for sequences see Table 1). Additionally, the *Eip28/29* distal element contains an imperfect DR3. As described above, this element is also targeted by both DHR78 and SVP homodimers (Zelhof et al., 1995b; Zelhof et al., 1995a). *Eip28/29* is expressed in a wide variety of tissues and at many times during development (Andres and Cherbas, 1992).
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The EcREs of two strictly stage- and tissue-specific genes have been characterized in the fat body of third instar larvae: the *fat body protein 1* (*Fbp1*) EcRE is localized upstream of the transcription start of *Fbp1*, which is expressed at the end of the third larval instar (Laval et al., 1993; Antoniewski et al., 1994). Also located in the upstream promoter region is the EcRE of the *larval serum protein 2* (*Lsp-2*) gene (Antoniewski et al., 1995). *Lsp-2* is expressed from the beginning of the third larval instar to the end of adult life. Both EcREs are arranged as palindromes (for sequences see Table 1).

EcR/USP binding sites have been also found in three of the *salivary gland secretion protein* (*Sgs*) genes. This group of seven genes encodes proteins which are needed for attaching the larva to the substrate preparative to pupariation. Expression of the *Sgs* genes is restricted to the salivary glands and coincides with the formation of the intermolt puffs in early to mid-third instar (for review see Lehmann, 1996). Several EcREs composed of two palindromic half-sites are present in the upstream regions of *Sgs-3* (Lehmann et al., 1997), *Sgs-4* (Lehmann and Korge, 1995), and *Sgs-8* (Hofman et al., 1991) (for sequences see Table 1).

Another gene mapping to an ecdysone-inducible intermolt puff, the *nested gene* (*ng*), contains an EcRE which is composed of AGGTCA half-sites arranged as DR elements (D'Avino et al., 1995). The *ng* EcRE can be regarded as a DR12, a degenerated DR2 or DR4. More interestingly, this is the only known EcRE that is capable of binding both EcR/USP and DHR38/USP heterodimers (Crispi et al., 1998). In addition, binding to this motif has been shown for USP alone, DHR38 alone, DHR39 and βFTZ-F1.

Studies using synthetic EcREs confirmed that binding of the EcR/USP complex is far more flexible than already suggested by the wide variability among natural EcREs (see above). Recently, it has been shown that EcR/USP prefers a PALI composed of two half-sites AGGTCA rather than DRs (e.g. DR4) or the *hsp27* EcRE for high affinity DNA binding and transactivation (Vögltli et al., 1998; Wang et al., 1998). Interestingly, these results were obtained with two different insect species, *D. melanogaster* and
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Aedes aegypti, respectively. Whereas the hsp27 EcRE was a relatively weak element in band shift experiments, Vögtli et al. demonstrated that it has only a slightly reduced transactivation potential compared to a perfect PAL1 (5'-GAGGTCAATGACCTC-3') when transiently transfected into Drosophila SL-2 cells (PAL1 > hsp27 EcRE = DR4). Using DR elements with one half-site different from AGGTCA, Antoniewski ct al. (1995) determined a similar order of decreasing binding affinities (DR4 > hsp27 EcRE) in band shift assays. However, this order was reversed when performing transactivation assays in Drosophila SL-2 cells.

1.9 Aim of the project

In order to get a better understanding of the mechanisms underlying ecdysone-dependent gene regulation on the level of DNA binding, we decided to isolate EcR/USP binding sites from the genome of two insect species, Drosophila melanogaster and Chironomus tentans, respectively. We have chosen Drosophila, because nearly all our knowledge about EcREs results from studies done in this insect species and because of the well characterized genetics. Furthermore, we wanted to confirm or disprove the findings of Vögtli et al. (see above) who defined a perfect PAL1 as the optimal binding site, which is in contrast to the so far characterized EcREs of Drosophila, which represent mostly degenerated PALs. Chironomus was chosen because nothing at all is known in this species about the structure of putative EcREs. In addition, studies on the polytene chromosomes can be performed much easier due to the size of the chromosomes.

A comparison of the identified EcREs should reveal whether these motifs are conserved among different insect species. The ultimate aim of such studies is of course the identification of the corresponding genes and the determination of their role in the ecdysone-regulated hierarchy.
2. MATERIALS AND METHODS

2.1 Animals and cell culture

*Chironomus tentans* strain 2Lk, *Drosophila melanogaster* wildtype strain OregonR as well as *Drosophila melanogaster* strain yellow/white were maintained according to standard methods. The *D. melanogaster* cell line SL-2 was cultured in Schneider's medium (Gibco-BRL) containing 10 % fetal calf serum and 100 U/ml penicillin/streptomycin as supplement.

2.2 Solutions and media

**TE** 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

**TBE** 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3

**TAE** 40 mM Tris-acetate, 1 mM EDTA, pH 8.0

20 x SSC 3 M NaCl, 0.3 M sodium citrate, pH 7.0

**PBS** 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.3

**Binding buffer (EMSA, whole genome PCR)**

20 mM HEPES-KOH, 100 mM KCl, 2 mM DTT, 5 % glycerol (v/v), 0.1 % NP-40, pH 7.4

**Glancy** 90 mM KCl, 40 mM NaCl, 20 mM triethanolamine, pH 7.6
Materials and Methods

NKE 100 mM NaCl, 5 mM KCl, 10 mM Tris-maleate, 0.5 mM EDTA, pH 6.3

Hybridization buffer (in situ hybridization)
5 x SSC, 0.1 % (w/v) SDS

Hybridization buffer (Southern blot)
5 x SSC, 50 % formamide, 0.1 % (w/v) N-lauroylsarcosine, 0.02 % (w/v) SDS, 2 % Blocking Reagent (Boehringer Mannheim)

Washing buffer 1 (Southern blot)
2 x SSC, 0.1 % SDS at RT

Washing buffer 2 (Southern blot)
0.1 x SSC, 0.1 % SDS at 68°C

SOC medium (1 liter)
2.0 g Bacto-tryptone (Difco), 0.5 g yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 0.36 % glucose

SOB medium (1 liter)
20.0 g Bacto-tryptone, 5.0 g yeast extract, 10 mM NaCl, 2.5 mM KCl

LB medium (1 liter)
10.0 g Bacto-tryptone, 5.0 g yeast extract, 10.0 g NaCl

2.3 Plasmid constructs

Plasmids expressing EcR and USP for in vitro transcription/translation were kindly provided by M. Vögtli (pSCT-constructs, Vögtli et al., 1998) and J.-F. Mouilllet (pcDNA3-constructs; unpublished). Construction of pcDNA3 expression vectors
Materials and Methods

The corresponding cDNAs encoding the three dEcR isoforms were amplified in a PCR reaction using as reverse primer 5'-ACCTCTCTAGACTATGCAGTCGAGTGGTC-3' together with the forward primers 5'-CACCCGGATCCACCATGTGACGACGGATGGA-3' for dEcR-A, 5'-CACCCGGATCCACCATGAAGCGGGCGCTGGAAC-3' for dEcR-B1, and 5'-CACCCGGATCCACCATGACTTTGTGGGATTAGTA-3' for dEcR-B2. The amplified fragments were cloned into the pcDNA3 vector as BamHI/XbaI fragments. Similarly, the expression vector pcDNA3-dUSP was generated by PCR amplification of the corresponding cDNA with the two primers 5'-TACCCGAATTCCACTGGACAACTGCACCAGGAC-3' and 5'-ACCTCTCTAGACTACTCCAGTTTCATCGCCAG-3' followed by subcloning as EcoRI/XbaI fragment into pcDNA3. Plasmid pSP-PAL1/AT(8x), which contains four tandem repeat copies of the synthetic PAL1 motif (Vögli et al., 1998) was a gift from M. Vögli.

2.4 Oligonucleotides

The following oligonucleotides were purchased from Microsynth:

- PCR-linkerA: 5'-GATCAGAAGCTTGAATTCGAGCAG-3'
- PCR-linkerB: 5'-CTGCTCGAATTCAAGCTTCT-3'
- PAL1/A: 5'-GATCTAGAGGGTCAATGACCTCGTCCAAG-3'
- T7-primer: 5'-TAATACGACTCACTATAGGG-3'
- T3-primer: 5'-AATTAACCCTCACTAAAGG-3'
- SP6-primer: 5'-GATTTAGGTGACACTATAG-3'

2.5 Antibodies

The monoclonal mouse antibody DDA2.7 raised against D. melanogaster EcR (Talbot et al., 1993) was a gift from D. Hogness. The monoclonal mouse antibody AB11
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(Christianson et al., 1992) raised against *D. melanogaster* USP was a gift from F. Kafatos. This antibody crossreacts with *C. tentans* USP.

2.6 Agarose gel electrophoresis and fragment isolation

Separation of DNA by agarose gel electrophoresis was performed according to standard methods using TAE. Fragments were isolated using either the QIAquick Gel Extraction Kit (Qiagen) or the AgarACE Agarase-Digesting Enzyme (Promega) following the suppliers protocol.

2.7 Cloning and plasmid isolation

Standard methods for manipulating DNA were as described by Sambrook et al. (1989).

2.8 Whole genome PCR

For *C. tentans*, genomic DNA was isolated from whole prepupae, for *D. melanogaster*, genomic DNA was isolated from the tissue culture cell line SL-2 according to the protocol I given by Sambrook et al. (1989). Genomic DNA was digested with *Sau3A* following standard methods (Sambrook et al., 1989). To allow subsequent PCR amplification, DNA fragments were ligated to specific adapters which were generated by annealing of PCR-linkerA (5'-GATCAGAAGCTTGATTGTAGCTGAG-3') and PCR-linkerB (5'-CTGCTCGAATTCAAGCTTCT-3') (Saunders et al., 1989). PCR-linkerB was used as primer for further PCR amplifications. Ligation products were separated on 2 % agarose gel and fragments from 250 bp to 750 bp were purified. To increase the amount of DNA, PCR was performed as described (Orlando and Paro, 1993). For the first round of whole genome PCR, 1 µl of each bacterially expressed, purified EcR and USP proteins (generously provided by C. Elke, Elke et al., 1997) were incubated in a final volume of 100 µl binding buffer containing 12 mg/ml BSA (final concentration),
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$10^5$ M muristerone A (final concentration) (Sigma), and 12 $\mu$g poly[dl-dC] to block unspecific binding. After 15 min on ice, 1 $\mu$g of adapter-modified genomic DNA was added. The reaction was further incubated for 45 min at RT with the addition of 2 $\mu$l of anti-dUSP antibody AB11 after 15 min. The formed immunocomplexes were recovered by the use of magnetic beads (Magnetic Cellulose Conjugated to Sheep Anti Mouse IgG, ANAWA) which were prewashed in binding buffer (12 mg/ml final concentration BSA) as recommended by the supplier. The beads were washed three times in binding buffer and the selected DNA was recovered by heating the beads at 65°C twice for 15 min in 200 $\mu$l TE, 1 % SDS (w/v) (Koelle et al., 1991). The eluted DNA was precipitated and used as template for 16 cycles of PCR amplification as described above. PCR products were purified (Qiaquick PCR Purification Kit, Qiagen) and one half of the material was used for further rounds of selection/amplification. After six rounds of whole genome PCR, DNA fragments were cloned into pGEM-T (Promega).

2.9 Labeling techniques

2.9.1 DIG labeling via PCR

Probes for Southern blot analysis were generated by incorporation of Digoxigenin-11-dUTP during PCR according to the suppliers protocol (Boehringer Mannheim), using as templates genomic DNA fragments which were obtained with whole genome PCR. The cycling conditions were modified as follows: 50 $\mu$l reactions containing 1 $\mu$M PCR-linkerB (final concentration) as primer were run for 30 cycles with annealing at 55°C for 1 min. Labeled probes were purified on agarose gels (see above) to reduce non-specific hybridization through PCR by-products. Labeling efficiency was estimated in a spot test as described elsewhere (The DIG System User's Guide for Filter Hybridization, Boehringer Mannheim).
2.9.2 Random priming with biotin or DIG

Probes used for in situ hybridization (ISH) were labeled by a random priming reaction (Feinberg and Vogelstein, 1984) using biotin-16-dUTP or Digoxigenin-11-dUTP (Random primed DNA labeling kit, Boehringer Mannheim). A 20 μl standard assay contained 100 ng of heat denatured DNA as template. After incubation for 20 h at 37°C, the reaction was stopped by adding 2 μl 0.2 M EDTA and stored at -20°C. Labeled probes were used without further purification steps. For ISH with Chironomus only biotin labeled probes were used, ISH with Drosophila was performed with either biotin or DIG labeled probes.

2.9.3 End labeling with \[^{32}P\]

Probes used for gel shift assays were radiolabeled as described below: the annealed ds oligonucleotide PALI was end labeled with 1 u/μl Klenow fragment of E. coli DNA Polymerase I (Boehringer Mannheim) and 2 μl [α-\(^{32}\)P]dCTP (3000 Ci/mmol; 10 μCi/μl) (Amersham) in a 25 μl reaction for 15 min at 37°C. Genomic DNA fragments amplified by PCR were end labeled with 10 U/μl bacteriophage T4 polynucleotide kinase (T4 PNK) (New England Biolabs) and 2 μl [γ-\(^{32}\)P]ATP (5000 Ci/mmol; 10 mCi/ml) (Amersham) in a 20 μl reaction for 45 min at 37°C. Unincorporated nucleotides were removed using Sephadex G25 columns (Boehringer Mannheim).

2.10 Sequence analysis

Sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase 2.0 sequencing kit (Amersham) with T7 and SP6 as primers. Analysis was carried out with the programs DNA Strider™ 1.2 and GCG 9.0 (University of Wisconsin), respectively.
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2.11 In vitro transcription and translation

Transcription/translation of cDNAs encoding EcR and USP was performed using the TNT® T7 coupled rabbit reticulocyte lysate system according to the manufacturer's protocol (Promega).

2.12 Electrophoretic mobility shift assay (EMSA)

For a 20 µl binding reaction, 1 µl of programed lysate or 2 µl of unprogramed lysate were incubated on ice for 15 min in binding buffer containing 1 µg poly[dI-dC] and, as a rule, muristerone A (10⁻⁵ M final concentration) (Sigma) unless indicated otherwise in the text. After the addition of [³²P] labeled DNA (max. 2 pmol/sample) the reaction mixture was left for 20 min at RT. For competition experiments, various amounts of unlabeled DNA were added together with the radiolabeled probe. When performing supershift assays, 1 µl of AB11 was given 10 min after the probe was added. Protein-DNA complexes were separated from free DNA at 150 V on a 4 % non-denaturing polyacrylamide gel in 0.5 x TBE, that was prerun for 2 h at 100V. The gels were dried and incubated for autoradiography at -80°C. Normally, overnight exposure was sufficient to visualize the signals. Unless otherwise mentioned, the pSCT-constructs (cEcR and cUSP-2 for Chironomus, dEcR-B1 and dUSP for Drosophila) were used for EMSA.

2.13 Southern blot analysis

Using standard methods (Sambrook et al., 1989), total genomic DNA from C. tentans and D. melanogaster was completely digested with different restriction enzymes as indicated in the text. After precipitation, the resuspended DNA was fractionated in 1 x TBE by 0.7 % agarose gel electrophoresis at 4°C and 1 V/cm. The following steps were performed at RT as recommended (The DIG System User's Guide for Filter
Hybridization, Boehringer Mannheim). Subsequently, depurination, denaturation, and neutralization were performed as described. The gel was blotted overnight to a nylon membrane (Boehringer Mannheim Nylon Membrane, positively charged) by capillary transfer using 20 x SSC and the DNA was fixed to the membrane through baking for 30 min at 120°C. The blot was then prehybridized for 1 h at 42°C in hybridization buffer, followed by hybridization to denatured DIG labeled probes overnight at 42°C in hybridization buffer (5 ng of labeled probe/ml hybridization buffer). The membrane was washed under stringent conditions with washing buffer 1 and 2 as recommended in the manual (see above) and detection of the probe was performed using anti-DIG-AP (Boehringer Mannheim) and CSPD® (Boehringer Mannheim) as chemiluminescent substrate. Prior to reprobing, the membrane was rinsed in H₂O and stripped by incubating twice for 10 min at 37°C in 0.2 N NaOH, 0.1 % SDS.

2.14 Chromosome in situ hybridization

*Chironomus tentans* salivary gland polytene chromosomes were isolated as described (Hertner et al., 1986). Preparation of *D. melanogaster* polytene chromosomes from third instar larvae maintained at 18°C followed a slightly modified protocol: salivary glands were dissected in NKE, transferred to a siliconized slide, incubated for 30 s in Glancy, and were finally fixed for 5 min in 50 % acetic acid. The chromosomes were squashed, frozen for 10 min on dry ice, and immediately washed twice for 10 min in 100 % EtOH. The conditions for indirect fluorescence in situ hybridization (FISH) and immunodetection were the same for both species. Briefly, squashed chromosomes were denatured at 70°C for 5 min in 70 % formamide, 2 x SSC and hybridized to denatured biotin labeled probes diluted in hybridization buffer at 58°C overnight in a moist chamber. For signal detection, we used as first antibody a mouse anti-biotin antibody (Boehringer Mannheim) diluted 1:20 and as secondary antibody a rhodamine-coupled anti-mouse cyb3 in a 1:1000 dilution. Antibodies were diluted in PBS, 0.1 % Tween-20, 10 mg/ml BSA and incubated for 30 min in a humidified chamber at RT. Finally, the slides were mounted in glycerol and inspected with a fluorescence microscope using the
appropriate filter settings. To document the banding morphology and the stained loci, chromosomes were photographed (Ilford Delta 400). In addition to FISH analysis with biotin labeled probes, a different protocol for signal detection was carried out using *Drosophila* probes labeled with DIG: after hybridization (see above) a 1:200 dilution of peroxidase-conjugated anti-DIG FAB fragments (Boehringer Mannheim) was applied (dilution in PBS, 0.1 % Tween-20) and the slides were incubated for 30 min in a moist chamber at 37°C. For enzyme-based detection, slides were overlaid with staining solution (PBS, 0.5 mg/ml diaminobenzidine, 0.01% H₂O₂) and incubated for 1-10 min at RT. The reaction was stopped by rinsing in H₂O and the chromosomes were counterstained for 30 s with a 1:20 dilution of Giemsa stain in 10 mM Na₃PO₄, pH 6.8. Finally, the slides were mounted in D.P.X. (Fluka). The chromosomes were examined under the microscope (Axioplan, Zeiss) using phase-contrast optics (Neofluar, Zeiss) and the digital pictures (ProgRes 3008, Digital Camera) further processed (Adobe Photoshop 4.0).

2.15 Western blot analysis

A 10 % SDS-PAGE (Laemmli, 1970) was loaded with 1 μl of each in vitro translated EcR and USP proteins or 1 μl of unprogramed reticulocyte lysate, respectively. Known molecular weight standards (SDS-PAGE protein standards, broad range) (Biorad) were loaded as control. After running of the gel at 10 V/cm, proteins were transferred onto nitrocellulose membrane (Protan nitrocellulose BA85, 0.45 μm, Schleicher & Schüll) by electroblotting for 1 h at 0.8 mA/cm². Blots containing EcR proteins were incubated with the monoclonal mouse antibody DDA2.7 at a 1:200 dilution, blots containing USP proteins were probed with the monoclonal mouse antibody AB11 at a 1:3000 dilution. As secondary antibody we used a peroxidase-coupled goat anti-mouse antibody (Pierce) diluted 1:3000. All dilutions were performed in PBS, 5 % (w/v) nonfat dry milk, 0.3 % (v/v) Tween-20. Bands were visualized by chemiluminescence.
3. RESULTS

3.1 Isolation of EcR/USP binding sequences from *Chironomus tentans* and *Drosophila melanogaster*

To select for putative EcREs in the genome of *Drosophila melanogaster* and *Chironomus tentans*, we have chosen the whole genome PCR approach, which was used previously to isolate hormone response elements (Costa-Giomi et al., 1992; Caubin et al., 1994). In preliminary experiments the optimal conditions for immunoselection and PCR amplification had to be established. A BamHI/BglII fragment of pSP-PAL1/AT(8x) containing 8 copies of PAL1 was ligated to adapters and incubated together with *in vitro* translated EcR/USP in a binding reaction as described in Materials and Methods. Although these experiments were successful (data not shown) we failed when using adapter-modified genomic DNA instead of plasmid DNA. We assumed that this could be due to the low amount of receptor proteins in the respective programed lysates. Therefore, we changed to bacterially expressed, purified EcR and USP (Elke et al., 1997) as protein source for our whole genome PCR approach. A summary of the procedure is schematically outlined in Figure 3A. Binding reactions were routinely performed in the presence of $10^{-5}$ M muristerone A to exclude the selection of binding sites for USP alone, which has been reported by several authors (Antoniewski et al., 1995; D'Avino et al., 1995; Vögtli et al., 1998; Wang et al., 1998). In addition, this was important since we used the anti-dUSP antibody AB11 for immunoprecipitation of protein-DNA complexes due to its high affinity binding to both dUSP and cUSP, respectively. Genomic adapter-modified DNA was subsequently incubated with EcR/USP and AB11. The formed immunocomplexes were captured with magnetic beads (see Materials and Methods), the DNA was separated from the beads and finally used as template for PCR amplification (Fig. 3A). The number of sixteen PCR cycles was determined experimentally. Aliquots were taken after several numbers of cycles and run on agarose gel in order to compare the selection/amplification of specific
fragments (i.e. reactions with AB11) to the amplification of unspecifically bound DNA, which was monitored by setting up control reactions containing no antibody: immunoselected DNA could be detected with sixteen cycles of PCR, whereas PCR products resulting from unspecifically bound DNA showed up after twenty cycles (not shown).

Totally, six rounds of whole genome PCR were performed. To initiate the analysis of selected DNA fragments we carried out competition band shift assays with PAL1/AT (referred to here as PAL1) as radiolabeled binding motif (Vögli et al., 1998) and the selected/amplified DNA resulting from round 1 to round 6 of the selection process as competitors in a 40-fold molar excess. As depicted in Figure 3B for *Chironomus* and Figure 3C for *Drosophila*, three rounds of whole genome PCR were already sufficient to displace part of the bound probe. After five to six rounds, binding of EcR/USP was completely competed away from the DNA element. This result clearly demonstrates that we really selected for specific EcR/USP binding sites. The collection of genomic fragments from round 6 was subjected to further characterization.
Figure 3. Whole genome PCR to select for putative EcREs in the genome of *C. tentans* and *D. melanogaster*. (A) Scheme of the followed protocol. Digested genomic DNA was brought to a form suitable for PCR amplification by adapter ligation. Fragments of a defined size were incubated with bacterially expressed EcR/USP in the presence of muristerone A (10⁻⁵ M) and subjected to six rounds of selection/amplification. Enrichment of EcR/USP binding sites through the selection process was confirmed by competition EMSA using *in vitro* translated EcR/USP and [³²P] labeled PAL1/AT (5'-GATCTAGAGGAGTCAATGACCTCGTCCAAG-3') (0.05 pmol, ~10⁴ cpm) (gelrun: 1.5 h). (B) Competition EMSA with selected DNA of *C. tentans* as competitors in a 40-fold molar excess as indicated on top of each lane. Unselected, adapter-modified genomic DNA is marked with 0. DNA resulting from each round of whole genome PCR is marked with 1 to 6. (C) Competition EMSA using selected DNA of *D. melanogaster* in a 40-fold molar excess. The triangle marks unspecific binding to the probe. For further explanation see (B).
3.2 Screening of individually cloned fragments obtained by whole genome PCR for EcR/USP binding

The DNA fragments from either C. tentans and D. melanogaster selected by six rounds of whole genome PCR were cloned directly after PCR amplification into the T-tailed vector pGEM-T. Transformants were plated on LB plates containing X-gal to monitor integration of an insert into the plasmid. White colonies were picked and plasmid DNA was isolated. In order to determine the size of the corresponding inserts, plasmids were digested with EcoRI to release the insert. This was possible since all inserts contain adapter sequences on both sites (see Materials and Methods) with restriction sites for EcoRI. In contrast, the pGEM-T vector carries no recognition sites for this restriction enzyme. The digestions were separated on agarose gel (not shown).

From 100 colonies tested, only 69 clones from C. tentans and 58 clones from D. melanogaster, respectively, contained recombinant plasmids. Next, we asked whether all of these clones would be able to compete against PAL1 binding to the EcR/USP heterodimer. Therefore we set up band shift assays using in vitro translated proteins and radiolabeled PAL1 together with the individually cloned fragments as cold competitors as described above. Examples of these competition EMSAs are shown in Figure 4. The intensity of the resulting shifts was compared to uncompeted binding (lane 2), self-competition with cold PAL1 (lane 3), competition with the DNA collection of round 6 prior to cloning (lane 4), and competition with the empty vector pGEM-T (lane 5). Approximately 45% of tested clones were able to displace binding of EcR/USP to PAL1, however more or less efficiently. Based on these findings we grouped the selected fragments into four classes according to their ability to compete with PAL1 (Table 2). For further investigations we focused on clones of class I (100% competition) and class II (partial competition).
Figure 4. Binding of individually cloned fragments to EcR/USP in competition EMSAs. *In vitro* translated proteins were incubated with [35S] labeled PAL1 (0.05 pmol, ~10^5 cpm) and individual clones as cold competitors for EMSA analysis. Only one example of performed competition EMSAs for *C. tentans* and *D. melanogaster*, respectively, is shown (gelrun: 1.5 h). Addition of different competitors is indicated above each lane. Except for the cold PAL1 (10-fold molar excess), competitors were added in a 40-fold molar excess. Round 6 is the selected DNA obtained with six rounds of whole genome PCR but uncloned, pGEM-T is the empty plasmid. Signals resulting from unspecific binding to the probe are indicated by a triangle. (A) Competition EMSA of clones from *D. melanogaster*. Probe bound to EcR/USP (arrow with solid head) is indicated. (B) Competition EMSA of clones from *C. tentans*. Probe bound to EcR/USP+AB11 (arrow with open head) is indicated. Note that the signal of bound probe to EcR/USP runs at the same position as unspecifically shifted probe. Therefore, supershift experiments with AB11 were performed. For sequence of PAL1 see Figure 3.
Table 2. Summary of screening of individually cloned fragments by EMSA (Fig. 4)

<table>
<thead>
<tr>
<th></th>
<th>C. tentans</th>
<th>D. melanogaster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Class II</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Class III</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Class IV</td>
<td>40</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>57</td>
</tr>
</tbody>
</table>

The classification of clones is based on their ability to compete for EcR/USP binding to PAL1. Class I indicates total competition (Fig. 4A, lanes 9, 18 and Fig. 4B, lanes 6, 8, 11, 12, 15, 16, 17, 19), class II indicates partial competition ability (Fig. 4A, lane 7 and Fig. 4B, lane 18), class III means very weak competition ability (Fig. 4A, lanes 13, 16, 20 and Fig. 4B, lanes 9, 14), and clones showing no competition at all are grouped in class IV (Fig. 4A, lanes 6, 8, 10, 11, 12, 14, 15, 17, 19 and Fig. 4B, lanes 7, 10, 13). Total means the number of clones tested for each species in competition EMSA.

3.3 Sequence analysis of individually cloned fragments to define putative EcREs

Natural EcREs characterized so far are mostly composed as imperfect palindromic sequences, e.g. EcREs of hsp27, Fbp1, Eip28/29, and Sgs-4 (Lehmann and Korge, 1995, and references therein). There are only a few examples described of natural EcREs arranged as direct repeats, e.g. the ng EcRE (D’Avino et al., 1995) or the distal element of the Eip28/29 EcRE (Cherbas et al., 1991). Hence, it was interesting to find out whether our selected clones contained such motifs and whether the structure of EcR/USP binding sites is conserved between different insect species.
Results

We started sequencing the individually cloned fragments of class I and II (Table 2) by using either T7 or SP6 as primers. Unfortunately, it turned out that 65% of sequenced fragments from Chironomus and 56% of sequenced fragments from Drosophila, respectively, contained pSP-vector sequences with or without several copies of the synthetic PALI, suggesting a contamination with pSP-PALI/AT(8x) that was used previously to establish the method of whole genome PCR. These clones were discarded.

After discarding of duplicates, the sequences from six independent cloned fragments from C. tentans and four from D. melanogaster were determined on both strands and analyzed in detail (Fig. 5 and 6). The sequence length varies from 228 nt (17c) to 538 nt (7d), which was expected due to our experimental settings for the selection approach.

By comparing the nucleotide sequences using the DNA-Strider™ 1.2 program we noted that fragment 38d is an internal part of 7d, the latter contains an internal Sau3A restriction site (Fig. 6). Despite this finding both 38d and 7d were considered as independent clones. Next, we scanned the sequences for the existence of putative EcREs. Surprisingly, none of the so far characterized EcREs could be identified within the selected fragments. However, single half-sites of several natural EcREs were found in all of the clones (Fig. 5 and 6; Table 1). A search in the database (BCM Search Launcher, BLASTN) revealed no significant alignments with known sequences except for the Drosophila clone 42d, which is in part identical to rRNA genes from various species (Fig. 5). The characteristic features of each fragment are described below.

In the 340 nt sequence of Chironomus clone 42c, a half-site of the hsp27 EcRE, the single half-site ATTTCA of the Sgs-8 element (Hofman et al., 1991), and half-sites TGAACT of Eip28/29 distal element and AGGT/TA of Eip28/29 upstream element (Cherbas et al., 1991) are represented. In addition, 42c is the only Chironomus clone with an internal Sau3A restriction site (Fig. 5).

Chironomus clone 55c (258 nt) harbors half-sites of the Sgs-4 element II, the Sgs-8 element, TCACTT of Eip28/29 proximal element (Cherbas et al., 1991), and a single half-site of the FbpI EcRE (Fig. 5).
Results

Like clone 55c, the 285 nt long *Chironomus* fragment 57c contains half-sites of the *Sgs-8* element, *Sgs-4* element II, *Eip28/29* proximal element, and the *Fbp1* EcRE. Additionally, this clone contains the half-site TCAATG of *Sgs-3* element II (Lehmann et al., 1997) (Fig. 5).

In the *Chironomus* fragment 83c of 284 nt we detected a half-site of the *Sgs-8* element and the sequence CCGTCA, which is a half-site of the *Lsp-2* EcRE (Antoniewski et al., 1995) as shown in Figure 5.

The 228 nt long *Chironomus* fragment 17c contains the single half-sites GGGTCA of *Sgs-3* element I (Lehmann et al., 1997), TGAATT of the *Fbp1* EcRE (Antoniewski et al., 1995), AGTTTA of *Sgs-4* element II (Lehmann and Korge, 1995), and the canonical hexameric sequence AGGTCA (Fig. 5).

Clone 32c from *Chironomus* contains a half-site of *Sgs-3* proximal element, GTTTCA (Lehmann et al., 1997). In addition, we found the half-site TGCAGT of the *hsp27* EcRE (Riddihough and Pelham, 1987) in the 231 nt sequence (Fig. 5).

Besides the canonical sequence AGGTCA, the 447 nt long fragment 32d from *Drosophila* contains several half-site sequences, e.g. TGGGGT of *Eip28/29* distal element (Cherbas et al., 1991), single half-sites of the *Sgs-8* element, the *Sgs-3* proximal element, the *Sgs-3* element I and AGTTTCG, which is a half-site of *Sgs-4* element I (Lehmann and Korge, 1995). A single half-site of the *hsp27* EcRE could be also identified (Fig. 5).

In the 386 nt of *Drosophila* clone 42d there is only a single half-site of *Sgs-8* element present. Interestingly, this site maps to the part of the fragment which is identical to rRNA genes (Fig. 5).
Results

As evident from the alignment for the *Drosophila* clones 38d (271 nt) and 7d (538 nt), a half-site of *Eip28/29* upstream element and several copies of AGGTCA are represented in both fragments (Fig. 6). Three of these AGGTCA half-sites are arranged as direct repeats and could be read either as DR8 or as two interlocked DR1 motifs. Furthermore, clone 7d contains half-sites of *Sgs-4* element II, *Sgs-3* proximal element, the *Lsp-2* EcRE and two single half-sites of the *Fbp1* EcRE.

Figure 5 (next two pages). Complete nucleotide sequences of cloned fragments selected by whole genome PCR. Individual clones were sequenced from both sites using T7 and SP6. Clones 17c, 32c, 42c, 55c, 57c, and 83c derive from *C. tentans*, clones 32d and 42d derive from *D. melanogaster* as indicated on the left. Only one strand of the nucleotide sequence determined by either T7 (32c, 42c, 57c, 32d) or SP6 (17c, 55c, 83c, 42d) is shown (DNA-Strider™ 1.2 program). *Sau3A* restriction sites are marked with blue color, homologies to single half-sites of known EcREs are as follows (for references see text). *Sgs-8* element (green letters), *Sgs-3* element I (plain line), *Sgs-3* element II (pink), *Sgs-3* proximal element (dashed line), *Sgs-4* element I (dotted line), *Sgs-4* element II (blue letters), *Eip28/29* upstream element (red letters), *Eip28/29* proximal element (wavy line), *Eip28/29* distal element (small letters), *hsp27* EcRE (double line), *Fbp1* EcRE (bold line), and *Lsp-2* EcRE (grey). The canonical sequence AGGTCA (bold letters) and sequence identity to rRNA genes are indicated (italic letters). For the complete nucleotide sequence of EcREs see Table 1.
Results

42c:
1  AATCTATGAGAAATTTATTTTATTATTATTTACTATCGAGTTTTTTTTGGCG
61  CAAAAAGATCAAGAAATAAGTCGAGTACAATACGACAGAACCTCAAATAAT
121  AGATACAAATTTCTTTTTTTTTTTTTTTATAATTAGGGGTAGGGAATGG
181  GGGGTCTTTTgaaactTTTGATTTGCTTGTGGAATTTTTATCGACTTTTTAAAGATTC
241  TACCTTTTTAAAATTTGAGTTTTGAGGCTGGCCACAGGTTPCGACATGGGAACCA
301  CTTAGATGCCAGTCCATAATGAGCATCTCGGTTTATGC

55c:
1  AATCAATTTTCTAAATTTAAATTTAAATATCAATAAAATTTAATTGTAGTTTTTT
61  TCTTCAATTTTACCTAAATAGGGTTTTATTTATGCTTAAAATTTTTAATTTAAGT
121  TTTAAACTTTATTTAAAATATGTTTTTTTTTTTTTTAAATTTTTAAAATCTTTT
181  TTTATCACTTTAACTATTTTTTTATCGTTTGTGAATTTGATATAATTTTACATTAA
241  AAATTTAAAAAAATTATATC

57c:
1  GATGAAGTGTCAGCATCGGTCACGATTTGCTCTAAATAATTAAATAATGGATGAAATTTTA
61  ACAGATGAACATAAGAAAGCTATCGTCGATAACTGTTTATAGAATGAATAATAAT
121  TGAGAATATAAATATAAATAAAATAAATAAATAACTGCTCAGGCCCATTCTAAACCGCTCCA
181  AAAAAAAAAATGTTGAAAATAATTACAAAAAAATGGAAGGTTTTATTGAGAAAAAGTTTAT
241  TAATTAAAAAAAAATTATATTAGTTAAAAACTAAGCAAAAAAGATA

83c:
1  CATGAAAACAAAAATTTAAATAAAAAAAFTTTGCCAATTCTCGGCTCGTCA
61  CTATCCCTCTACAAATAAATTCTAATATGCTCTATCTGCGCCATTTCTAACCAGCTCCA
121  AACAAGCGCAAAACCTTAAATTCTCTACATTTTATCAAGGCTTACAATA
181  ATCAATTTAAAATACGAAGICAGTACTAGAGCTAAAATTCTCTCTTGGTGC
241  TAAAAATTAAAAAGGAATTTTATACACTTTCTGGAAGATA
Results

17c:

1  CTGACATCAAGCTCGTTGTTGTCAGGGAATGA
61  GTPAAATTGAAATAAAAATTTGGCTTAGAGAGCTAGTCAGTTCTTTATTCTTAGACAAA
121  TGATAAAAAAAAAGGAATATGAAATTTATATTACATCTGAATAAAAG
181  AAAACCAAAATAGTGTCCTTAATACACAAAATTTTTAATTTAAAAGATT

32c:

1  TATCGTAGACTTTGCATTTCTATATTTTTTTTCTATTACACAC
61  TTAACTTTTCAATTTTTATATTTGCACTATCGTTTATTTTTCTATCGATGC
121  AGCAACCGGGAAATATTTATTTTTTCTATTATGAATTTTCAATAAAATAGTC
181  ACAAGAAAGACCTTTGTCGGCAGCTGACGTTTTATCTAGATTCTCTGATTC

32d:

1  GATCGGAGGTATCTTTTTTTTTTTTTTTTTTGTAAGTTGTCGGAGCACAATTTATTTTC
61  TCCATTCCGCAGCGAGTTTCGAATAGTACAATATGATGACATACCAATGATTTCCC
121  AACTGATGGAATTATTATTCTTGTGAAATATTCTAGCAGTCCACCAGCTTAC
241  TGCGCCGACATATTTATATTTATTCAATAATTCCATTTTTTCGGCGCATTATTT
421  GATGGGTCAGCCCAGGAGGGTACCTCTCGCCCTCCCTCGAGAGAAATAGATTTCAAACCTATATGGGAAGA

42d:

1  TATCACCTCAGGGTGCCTACATGTGGAGGAGAAACATATTGATTTGGGTTT
61  TATTTTTTTTTTTTTTTTTTTTTTTTTTTATACAGAGACTCAGAATAATCTCAGCTTTTT
121  AGTCTAGCACAGGAGAAATATTGGTCTTTAGGCTCAGGCTG
181  TGTTGGAATGAGAGCCTACGTCTCTCTCGGTCTCTGTGGCAAGAGATAGTGCCCTCGGA
241  ACCCTCTTTTTCTTTCGTTATAGTTGAAAACACTCTCTTTGCTCTTAGTT
301  AGTCTTAGACGGGCTCTAGACCTCTCTCGCGCCATATTACCTGCCCACGCTC
361  CTCATTAATACAATACCTCCTTCTATGATTC
Figure 6. Nucleotide sequence comparison between fragments 7d and 38d from Drosophila. Alignment was done with the Pile Up program (GCG, University of Wisconsin). Only one strand of the sequence obtained by using SP6 as primer is shown. Restriction sites for Sau3A (blue) and Clal (red) are marked. The putative DR8/DR1 motif is indicated by the yellow color (for further explanation see text). For indication of single half-sites see legend to Figure 5.
3.4 EMSA studies of sequenced clones from Chironomus and Drosophila

So far, binding of our selected fragments to EcR/USP was only tested in an indirect way by using the fragments as competitors in band shift assays. In a second series of EMSA experiments, direct binding of these fragments to EcR/USP was compared to the competition ability of the corresponding fragments (Fig. 7). Competition EMSAs were carried out with the radiolabeled PAL1 and the individual clones in a 20 to 40-fold molar excess as described. The binding reactions for Chironomus were performed as supershifts with AB11 (anti-USP) in order to separate specifically shifted complexes from unspecific probe binding. The previously established classification into class I fragments (17c, 32c, 55c, 32d) and class II fragments (42c, 57c, 83c, 7d, 38d, 42d; for classification see Table 2) could be confirmed for both species (Fig. 7A and 7B). As depicted in Fig. 7A, the following order of decreasing competition ability was determined for Drosophila: 32d ≥ PAL1 > 42d ≥ 38d > 7d. The competition efficiency of the Chironomus fragments followed the order 17c = 32c = 55c > 83c > 42c > 57c. Concerning direct binding of the fragments to EcR/USP, nearly all of the binding reactions of either D. melanogaster (Fig. 7C) or C. tentans (Fig. 7D) revealed strong retarded bands. However, two clones did not follow the rule: the Drosophila clone 42d, which belongs to competition class II, has only a very low affinity to EcR/USP when used as labeled probe (Fig. 7C, lane 8). With clone 57c, we did not succeed to detect any direct binding to EcR/USP (not shown). This was unexpected since this fragment also belongs to competition class II. When looking at the binding pattern of 17c (Fig. 7D, lane 2) and 32c (Fig. 7D, lane 4), respectively, three shifted bands could be detected, indicating binding to these fragments of either EcR or USP monomers, heterodimers or multimers (see also Fig. 17). Taken together, these results confirmed that our selected fragments are indeed target sites for EcR/USP at least in our in vitro binding assays (EMSA).
Results

A.

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![Image A]

B.

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![Image B]

C.

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![Image C]

D.

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![Image D]
Figure 7 (previous page). EcR/USP binding to selected fragments. Binding affinities were tested by competition EMSA (A and B) as well as direct EMSA (C and D). Binding reactions were performed with in vitro translated EcR/USP as described. For competition EMSA, PAL1 (for sequence see Fig. 3) was used as $[^{32}\text{P}]$ labeled probe (0.05 pmol, $-10^4$ cpm). Signals resulting from unspecific probe binding (triangle) and specific binding to individual clones (open arrowhead) are indicated as well as the use of unprogramed reticulocyte lysate (-). (A) EMSA with Drosophila clones as competitors (20-fold molar excess). The arrow marks probe bound to EcR/USP (gelrun: 1.5 h). (B) EMSA with Chironomus clones as competitors (17c, 32c, 42c, 83c: 20-fold; 55c, 57c: 40-fold molar excess). AB11 was added to each reaction. The open arrow marks the position of supershifted complex bound to PAL1 (gelrun: 1.5 h). (C) EMSA with individual clones from Drosophila as $[^{32}\text{P}]$ labeled probes ($-10^4$ cpm). Lanes 1-4 contain 30 fmol, lanes 5-8 contain 0.05 fmol of labeled probe. Gels were run for 2 h (7d, 32d) and 3 h (38d, 42d). (D) EMSA with individual clones from Chironomus as $[^{32}\text{P}]$ labeled probes ($-10^4$ cpm). Except for lane 7 and 8 (1.9 pmol), all lanes contain 5 fmol of labeled probe. Gels were run for 2 h (55c), 2.5 h (17c, 83c) or 3 h (32c, 42c).
Next, we wanted to test whether the DR motif of 7d/38d is involved in EcR/USP binding. Therefore, clone 7d was digested with Clal and EcoRI which resulted in three internal fragments, two EcoRI/Clal fragments and one Clal/Clal fragment (note that both restriction sites are missing in the vector and that EcoRI cuts in the adapter). These fragments were subcloned into pBluescript® II SK+ (Stratagene) and named according to the original clone 7d: pBS-7d[1-159], pBS-7d[160-307] (containing the DR motif) and pBS-7d[308-538]. Subsequently, the subfragments were labeled with $^{32}$P as described and subjected to EMSA. As predicted, a clear shifted band was detected when using fragment 7d[160-307] (Fig. 14B, lane 2). The two other fragments were also able to bind to EcR/USP, however with much lesser affinity (Fig. 16, lanes 8, 9, 10).

3.5 Genomic Southern blot analysis

To show that our selected clones are really of genomic origin and to determine how many gene copies are present in the genome, we performed Southern blots with the individual clones as DIG labeled probes. Genomic DNA from C. tentans and D. melanogaster, respectively, was digested with either EcoRI, SalI, HindIII, or BamHI and subjected to Southern blot analysis (Fig. 8). Hybridization and rehybridization of stripped blots were as described (see Materials and Methods). All fragments were able to hybridize to genomic DNA, indicating that they are indeed of genomic origin. Genomic fragments 17c, 55c, 57c, 83c, and 38d (Fig. 8B, lanes 1, 6, 7, 8, 9 and Fig. 8D, lanes 5, 6) gave only one signal, suggesting single copy genes. Hybridization with probes 32c, 42c, 32d and 42d (Fig. 8B, lanes 2, 3, 4, 5 and Fig. 8D, lanes 3, 4, 7, 8) resulted in a smear possibly due to repetitive sequences. With probe 7d two signals could be detected (Fig. 8D, lanes 1, 2) suggesting the existence of two gene copies.
Figure 8. Genomic Southern blot with selected fragments as DIG labeled probes. Genomic DNA from *C. tentans* and *D. melanogaster*, respectively, was digested as indicated above each lane (*E. EcoRI; S, SalI; H, HindIII; B, BamHI*), separated on 0.7 % agarose gel (TBE), blotted to nylon membrane and subjected to hybridization. Probes used are indicated at the bottom of each strip. Positions of signals obtained by detection with CSPD® were compared to a standard (*M, 1 kb ladder, Gibco-BRL*). (A) Agarose gel with digested DNA from *C. tentans* and (B) hybridized blots after signal detection. (C) Agarose gel with digested *Drosophila* DNA and (D) hybridized blots after signal detection.
3.6 Mapping of selected genomic fragments to chromosomal loci

In situ hybridization is a useful tool to investigate the chromosomal localization of DNA sequences, especially on the giant polytene chromosomes from *C. tentans* and *D. melanogaster*. Therefore, squashed preparations of polytene chromosomes were hybridized with biotin labeled fragments. In addition, *Drosophila* chromosomes were hybridized with DIG labeled fragments (see below). The hybridized fragments were detected by indirect immunofluorescence (biotin labeled probes) or by an enzyme-based reaction (DIG labeled probes) followed by a detailed characterization of stained loci.

3.6.1 Localization studies in *Chironomus tentans*

*C. tentans* has four pairs of chromosomes. Although there are only a few genes characterized, transcriptional activity often represented by puffs (Clever and Karlson, 1960; Clever, 1964; Pelling, 1964) as well as localization of endogenous EcR/USP with the respective antibodies (Wegmann et al., 1995) were objects of detailed examinations. In a first series of experiments we tested the selected but uncloned collection of DNA obtained by six rounds of whole genome PCR (Fig. 3) as probes. After immunodetection, the fluorescence signals were mapped according to Pelling (1964) (mapping by M. Lezzi). Surprisingly, we obtained only a few but prominent signals spread over the four chromosomes (Fig. 9 and Table 3). Stained loci II-14A (panel d), IV-2B and (panel h) could be correlated to previously described ecdysone-inducible puff-sites. Locus IV-2B encodes cE75, locus II-14A cUSP (Wegmann et al., 1995), whereas the second signal IV-5C detected on chromosome IV maps to a so-called heat shock puff (Lezzi et al., 1981; Sass, 1982) and it is decorated with endogenous EcR/USP only after heat shock (Lezzi, 1996). Concerning the stained loci on chromosome I (I-7-8; panel b) and III (III-15; panel f), there is nothing known about ecdysone inducebility. In summary, these results provide the first hints that at least some of our selected fragments are associated with known ecdysone-inducible loci.
Figure 9. *In situ* hybridization to *Chironomus* polytene chromosomes probed with uncloned DNA which was selected by six rounds of whole genome PCR. Detection of biotin labeled probes was performed with anti-biotin and a rhodamine-conjugated secondary antibody (indirect FISH). The biotin labeled probe (on the left) and the corresponding chromosome (on the right) are indicated. Left panels show the phase contrast, right panels the fluorescence signals with filter for rhodamine, both using a 100x objective lens. Stained loci are marked with the arrows. (b) Locus I-7-8. (d) Locus II-14A. (f) Locus III-15. (h) Loci IV-2B (1) and IV-5C (2).
Results

In order to substantiate these data, we subjected the individually cloned fragments 17c, 32c, 42c, 55c, 57c, and 83c to FISH analysis as described above. The resulted staining pattern shown in Figure 10 was very unexpected. None of the previously stained loci could be detected with any of the probes. Nevertheless, some of the detected signals could be mapped to known puff-sites (Fig. 10 and Table 3). Both signals obtained with probe 42c can be localized to described puff-sites, namely locus I-20A on chromosome I and locus II-19B2 on chromosome II (Fig. 10A, panel d). The latter is also stained by probe 55c (locus II-19B; Fig. 10A, panel f) and probe 57c, in addition to staining of II-19A (Fig. 10B, panel a). Probes 17c (Fig. 10A, panel b) and 83c (Fig. 10B, panel f) hybridize to non-characterized loci, probe 32c most probably hybridizes to or close to the centromer of chromosome I (Fig. 10B, panel d). Results from Chironomus FISH are summarized in Table 3.

Table 3. In situ hybridization to C. tentans chromosomes

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<th>Relevance</th>
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<tr>
<td>I-7-8</td>
<td>puff, cUSP locus</td>
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</tr>
<tr>
<td>II-14A</td>
<td>puff-site</td>
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<tr>
<td>III-15</td>
<td>puff, cE75 locus</td>
<td></td>
</tr>
<tr>
<td>IV-2B</td>
<td>HS puff</td>
<td></td>
</tr>
<tr>
<td>IV-5C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17c</td>
<td>III-7B/C</td>
<td>/</td>
</tr>
<tr>
<td>32c</td>
<td>I-10</td>
<td>~ centromer</td>
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<tr>
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<td>HS puff</td>
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<td>II-19B</td>
<td>HS puff</td>
</tr>
<tr>
<td>57c</td>
<td>II-19A</td>
<td>HS puff</td>
</tr>
<tr>
<td>II-19B2</td>
<td>HS puff</td>
<td></td>
</tr>
<tr>
<td>83c</td>
<td>II-3</td>
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</tr>
</tbody>
</table>

Polytene chromosomes of C. tentans were hybridized to biotin labeled probes and the detected signals mapped to known loci (see text and Figures 9, 10). HS, heat shock.
Results

A.

Probe:  Phase: 100x  Rhod: 100x

17c

42c

55c
B.

Probe:  Phase: 100x  Rhod: 100x

57c

32c

83c
Figure 10 (this and previous two pages). In situ hybridization to *Chironomus* polytene chromosomes probed with individually cloned fragments. Detection of biotin labeled probes was performed with anti-biotin and a rhodamine-conjugated secondary antibody (indirect FISH) and then mapped. (A) and (B) Left panels show the phase contrast, right panels the fluorescence signals with filter for rhodamine, both using a 100x objective lens. Stained loci are marked with the arrows. (A) Signals obtained with probes 17c (panels a, b), 42c (panels c, d), and 55c (panels e, f). (b) Locus III-7B/C, (d) Loci I-20A (1) and II-19B2 (2). (f) Locus II-19B. (B) Signals obtained with probes 57c (panels a, b), 32c (panels c, d), and 83c (panels e, f) as marked on the left. (b) Loci II-19A (1) and II-19B2(2). (d) Locus I-10. (f) Locus II-3. (C) Map of the four chromosomes of *C. tentans* according to Pelling (for reference see text). Transcriptional active sites are indicated on top of each chromosome (brackets) as well as an inversion in chromosome II (Inv. 2L).
3.6.2 Localization studies in *D. melanogaster*

*Drosophila* has four pairs of chromosomes fixed together at the chromocenter: the X/Y sex chromosomes and the autosomes II, III, and IV. Chromosomes II and III are further subdivided in a left and a right arm (2L, 2R, 3L, 3R). The size of the *Drosophila* genome is about 170 million bp and it contains an estimated number of 12,000 genes (Miklos and Rubin, 1996). In contrast to *C. tentans*, a large number of *Drosophila* genes are already characterized. For a genomic localization of our selected fragments, *in situ* hybridizations with the fragments as biotin labeled probes were performed as described (Materials and Methods). As expected, all four labeled probes hybridized to chromosomal loci (Fig. 11). With probe 7d (Fig. 11A, panel b) as well as with biotin labeled probes 7d[1-159] and 7d[160-307] (data not presented) we identified two signals close together on the same chromosome. Interestingly, only one signal was obtained when using 38d as probe, which is an internal part of 7d (see Fig. 6). Hybridization with either probe 32d (Fig. 11A, panel c) or probe 42d (Fig. 11B, panel d) also resulted in only one signal. In order to facilitate mapping of the stained loci, a modified protocol with DIG labeled probes followed by diaminobenzidine (DAB) staining (Materials and Methods) was used together with the *Drosophila melanogaster* strain yellow-white (collaboration with D. Nellen, Institute of Molecular Developmental Genetics 2, University of Zurich). The cytological positions of the detected signals were determined by comparison of the polytene bands to the map of Bridges (Lindsley and Zimm, 1992). As summarized in Table 4, the loci stained with probes 7d (X-chromosome, loci 12E and 13B; Fig. 11A, panel c) and 32d (chromosome 2R, locus 41F; Fig. 11A, panel f) could be precisely defined (mapping by D. Nellen). Unfortunately, we failed to detect a signal when performing *in situ* hybridization with probe 42d using the modified protocol (not shown). Therefore, we were not able to map the chromosomal locus stained with this probe. The only conclusion we can draw from our *in situ* hybridization experiments with 42d is that it does not hybridize to the locus harboring the gene cluster encoding the 18S, 5.8S, and 28S rRNA of *Drosophila*. This was unexpected since 42d is partially identical to these rRNA genes (see Fig. 5). In addition, it was not yet possible to determine exactly the stained locus of 38d, whether it
hybridizes to locus 12E or 13B on the X-chromosome. For the X-chromosomal locus 12E a correlation to an ecdysone-regulated puff could be shown (Ashburner, 1972b; Ashburner, 1975b). Due to its slightly delayed formation with regard to the late larval ecdysone pulse it belongs to the so-called early-late puffs (see Introduction). As shown by White et al. (1997), locus 13B also correlates to a puff-site. For the other mapped locus 2R-41F, which is located very close to the centromer, nothing is reported about puffing.

Table 4. In situ hybridization to D. melanogaster chromosomes

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<th>Cytological location</th>
<th>Relevance</th>
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<tr>
<td>7d</td>
<td>12E (X) 13B (X)</td>
<td>puffs</td>
</tr>
<tr>
<td>38d</td>
<td>12E (X) or 13B (X)</td>
<td>puffs</td>
</tr>
<tr>
<td>32d</td>
<td>41F (2R)</td>
<td>?</td>
</tr>
<tr>
<td>42d</td>
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</table>

Polytene chromosomes of D. melanogaster strain OregonR and yellow/white were hybridized to biotin and DIG labeled probes, respectively. The detected signals were mapped to chromosomal loci (see text and Fig. 11).
Results

A.

Probes: 7d

![Image 1]

Probes: 32d

![Image 2]
Figure 11 (this and previous page). *In situ* hybridization to *Drosophila* polytene chromosomes probed with individually cloned fragments. Detection of biotin labeled probes was performed with anti-biotin and a rhodamine-conjugated secondary antibody (indirect FISH). Detection of DIG labeled probes 7d and 32d were performed with anti-DIG and Diaminobenzidine (DAB), the chromosomes stained with Giemsa. The used probes are indicated on the top. Chromosomes are shown using a 100x objective lens. Panels (a) and (b): phase contrast. Panels (c) and (d): fluorescence signals with filter for rhodamine. Panels (e) and (f): DAB. Stained loci are marked with the arrows. Mapping was carried out according to the map of Bridges (for reference see text). (A) Signals obtained with probes 7d (panels a, b, c) and 32d (panels d, e, f). (b) and (c) Loci X-12E (1) and X-13B (2). (e) and (f) Locus 2R-41F. (B) Signals obtained with probes 38d (panels a, b), 42d (panels c, d). (b) Locus X-12E or X-13B.
3.7 Differentiation of EcR and USP binding to the selected fragments

Recent EMSA experiments done in our laboratory showing for the first time binding of all three *Drosophila* EcR isoforms alone to the synthetic element PAL1 and to a probe containing the *hsp27* EcRE, respectively (J.-F. Mouillet, personal communication), motivated us to test the binding ability of different EcR isoforms as well as USP alone to our selected fragments in the absence and presence of hormone.

Binding studies with the different isoforms of *Drosophila* EcR (dEcR) were performed with *in vitro* translated proteins expressed by pcDNA3-vectors carrying the corresponding cDNAs (see Materials and Methods). The *Drosophila* USP (dUSP) was also expressed as pcDNA3-construct when used in combination with each of the dEcR isoforms (pcDNA3-constructs). For comparison, also proteins expressed with pSCT-vectors were used. After *in vitro* transcription/translation, the amount of the respective receptor proteins in the programed lysates was estimated by Western blot analysis using DDA2.7 and AB11 as antibodies against dEcR and dUSP, respectively (Fig. 12). In general, the pSCT-constructs were less efficiently expressed compared to their pcDNA3 counterparts (compare lanes 2, 4 in Fig. 12A for dEcR-B1 and lanes 2, 3 in Fig. 12B for dUSP, respectively). Comparing the pcDNA3-constructs of the three dEcR isoforms, the expression level of EcR-B2 was highest, followed by EcR-A and B1, which only resulted in a faint band (Fig. 12A, lanes 3, 4, 5). As expected, no signals were detected in control reactions using unprogramed lysate (upl) with the two antibodies (Fig. 12, lane 1).
Results

A.

Figure 12. Expression levels of various dEcR and dUSP proteins produced by in vitro translation. 1 µl of each programmed lysate or unprogrammed lysate (upl) was separated by SDS-PAGE, blotted onto nitrocellulose and the blots were incubated with specific monoclonal antibodies as indicated at the bottom. Signal detection was carried out with chemiluminescence. The position of known protein standards is given on the left of each blot with the corresponding molecular weight. (A) Blot with different EcR isoforms and expression-constructs (indicated above each lane) probed with DDA2.7 (1:250 diluted). (B) Blot with different USP expression-constructs (indicated above each lane) probed with AB11 (dilution 1:3000).
Results

A. Results

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

B. Results

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

38d

7d

Free
Results

Figure 13. Binding affinities of EcR isoforms and USP to selected Drosophila fragments in EMSA. In vitro translated proteins expressed as pSCT- or pcDNA3-constructs were incubated with or without $10^5$ M muristerone A (Muri) and [$^{32}$P] labeled probes (5 fmol, ~ $10^4$ cpm) as indicated. Supershift experiments were performed with the addition of 1 μl AB11 to the binding reaction. The first lanes represent incubation of DNA fragments with unprogramed reticulocyte lysate. The following marks are indicated on the left: unspecifically bound probe (triangle), probe bound to the respective EcR/USP heterodimers or USP supershifts (bars), bound USP monomer (arrow with open head), and bound USP homodimers (arrow with solid head). Binding of EcR monomer is indicated on the right for EcR-A (solid arrowhead) and EcR-B1 (solid circle). The right panels are the results of longer exposure. (A) EMSA with 38d as labeled probe (gel run: 3 h). (B) EMSA with 7d as labeled probe (gel run: 4 h).
Results

For each EMSA reaction performed with labeled probes from *Drosophila*, the different dEcR isoforms alone or dUSP alone were incubated with the respective genomic fragment in the presence or absence of muristerone A (10⁻⁵ M) to test whether this kind of binding is hormone-dependent. Binding of USP alone to the probe was confirmed with supershift experiments by supplementation with 1 µl AB11. Reactions containing the various EcR/USP heterodimers were routinely incubated in the presence of 10⁻⁵ M muristerone A. As predicted, all three combinations of EcR/USP heterodimers revealed the strongest binding to the labeled probes (Fig. 13, 14, 15, 16). Concerning the binding affinity to EcR or USP alone, we identified two different groups of fragments which are specified below.

The first group comprising fragments 38d (Fig. 13A), 7d (Fig. 13B), 7d[160-307] (Fig. 14A), and 32d (Fig. 14B) share the following features. First, all possible heterodimers resulted in a strong shift, independently of the expression vectors (Fig. 13 and Fig. 14A, lanes 2, 8, 9, 10; Fig. 14B, lanes 2, 7, 8, 9). Second, no shifts were detected with dEcR-B1 alone expressed as pSCT-construct either in the presence or absence of ligand (see lanes 2, 3 of the respective Figures). This could be explained by the very low expression level of pSCT-dEcR-B1 (Fig. 12A, lane 2). Third, incubation with dUSP alone resulted in a prominent shift which was completely supershifted after the addition of AB11 (Fig. 13 and Fig. 14A, lanes 7, 19; Fig. 14B, lanes 6, 17). Due to the different expression levels of the two dUSP-constructs (Fig. 12B, lanes 2, 3) reactions containing the pcDNA-3 constructs showed slightly stronger shifted bands. In addition to the low molecular shift obtained with both dUSP-constructs and which indicates possible monomeric binding (Fig. 13 and Fig. 14A, lanes 5, 6, 17, 18; Fig. 14B, lanes 5, 16), binding of the pcDNA3-dUSP-construct to fragments 38d and 7d resulted in a second slower migrating band, maybe due to homodimeric binding (Fig. 13, lanes 17, 18). As expected, binding of USP alone to the genomic fragments was not affected by ligand (not shown for probe 32d).
Results

A.  

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Figure 14. Binding affinities of EcR isoforms and USP to selected Drosophila fragments in EMSA. In vitro translated proteins expressed as pSCT- or pcDNA3-constructs were incubated with or without $10^{-5}$ M muristerone A (Muri) and $^{[32P]}$ labeled probes (5 fmol, ~ $10^5$ cpm) as indicated. Supershift experiments were performed with the addition of 1 µl AB11 to the binding reaction. The first lanes represent incubation of DNA fragments with unprogramed reticulocyte lysate. For the marks on the left and on the right see Figure 13. (A) EMSA with 7d[160-307] as labeled probe (gelrun: 2.5 h). (B) EMSA with 32d as labeled probe (gelrun: 3.5 h). The right panel is the result of longer exposure.
In contrast to these common characteristics, the binding affinities of the different EcR isoforms (pcDNA3-constructs) are specific for each of the fragments. As depicted in Figure 13A, incubation of 38d with all three isoforms in the absence of ligand resulted in a more or less prominent retarded band (lanes 11, 13, 15, 20, 22, 24) indicating homodimeric binding which is very weak when hormone is present (lanes 12, 14, 16, 21, 23, 25). Additionally, a faster migrating band was identified with binding reactions containing EcR-A alone and muristerone A maybe due to binding of an EcR-A monomer (lanes 12, 21; solid arrowhead on the right). Taking into account the different expression levels (Fig. 12A), we can conclude for probe 38d that (i) binding to EcR-A is stronger (~2 times) than to the B2 isoform and (ii) interpretation of shifts obtained with EcR-B1 is difficult because of its low expression level. Concerning binding of EcR-B2 alone, similar results were obtained with fragment 7d (Fig. 13B, lanes 15, 16, 24, 25). However, differences were detected with the other two isoforms. While probe 7d showed a relative weak formation of the high molecular weight band when incubated with EcR-A alone in the absence of hormone (Fig. 13B, lanes 11, 20), this fragment showed strong binding to a possible EcR-A monomer in the presence of ligand (Fig. 13B, lanes 12, 21; solid arrowhead on the right). The most striking difference between fragments 38d and 7d is the strong binding of EcR-B1 to 7d, which is manifested in a prominent high molecular shift in the absence of ligand (Fig. 13B, lanes 13, 22) and a slower migrating band (monomeric binding) in the presence of hormone (Fig. 13B, lanes 14, 23; solid circle on the right). Thus, we can summarize that clone 7d shows relatively strong binding to EcR-B1 alone (despite the very low expression level of this isoform; see Fig. 12A, lane 4) which is higher than the binding affinity to the A isoform alone.
Results

Figure 15. Binding affinities of EcR isoforms and USP to the Drosophila fragment 42d in EMSA (gelrun: 3.5 h). In vitro translated proteins expressed as pSCT- or pcDNA3-constructs were incubated with or without $10^{-5}$ M muristerone A (Muri) and $[^{32}P]$ labeled probe (5 fmol, ~ $10^4$ cpm) as indicated. Supershift experiments were performed with the addition of 1 $\mu l$ AB11 to the binding reaction. The first lane represents incubation of the DNA fragment with unprogramed reticulocyte lysate. Unspecifically bound probe (triangle) and probe bound to the respective EcR/USP heterodimers (bars on the left) are indicated.
The EMSA experiments with the subfragment 7d[160-307], which contains the DR motif, resembles the pattern obtained with 38d, meaning also a strong retarded band with EcR-A alone in the absence of ligand (Fig. 14A, lane 11) and a faint faster migrating band with the same isoform when ligand was added (Fig. 14A, lane 12). However, this probe shows a much weaker binding to EcR-B2 (Fig. 14A, lanes 15, 16) than already mentioned for probe 38d (see above). Again, interpretation of the binding affinity to EcR-B1 is not possible due to its low expression level. The preference for the EcR-A isoform holds also true for probe 32d, as shown in Figure 14B (lanes 10, 11, 18, 19), although here the binding of EcR-A alone is very weak. With this probe we were not able to detect any binding to the EcR-B isoforms alone, either with or without hormone (lanes 12, 13, 14, 15, 20, 21, 22, 23).

The second group contains the genomic fragment 42d (Fig. 15) and the two subfragments 7d[1-159] (Fig. 16A) and 7d[308-538] (Fig. 16B). These clones do not bind to any EcR isoform alone but rather to all three possible EcR/USP heterodimers expressed as pcDNA3-constructs. For probe 42d, this is in agreement with previous findings where this fragment showed a weak binding affinity to EcR/USP in direct EMSA (Fig. 5C, lane 10). When comparing the binding intensity of heterodimers formed by EcR-A and EcR-B2, respectively, it seems that all of the three fragments have a slight preference for heterodimers composed of EcR-A/USP (note that the expression level of EcR-B2 is higher than that of EcR-A). Binding of EcR-B1/USP heterodimers is weaker with probe 42d compared to the other two fragments.
Results

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Free

7d[1-159]

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Free

7d[308-538]

Figure 16. Binding affinities of EcR isoforms and USP to subfragments of *Drosophila* clone 7d in EMSA. *In vitro* translated proteins expressed as pSCT- or pcDNA3-constructs were incubated with or without 10^{-3} M muristerone A (Muri) and [³²P] labeled probes (5 fmol, ~ 10⁶ cpn) as indicated. Supershift experiments were performed with the addition of 1 µl AB11 to the binding reaction. The first lanes represent incubation of DNA fragments with unprogramed reticulocyte lysate. Probe bound to EcR/USP heterodimers (bars) and unspecifically bound probe (triangle) are indicated. (A) EMSA with 7d[1-159] as labeled probe (gelrun: 2.5 h). (B) EMSA with 7d[308-538] as labeled probe (gelrun: 3 h).
Figure 17. Binding affinities of EcR and USP to selected Chironomus fragments in EMSA. *In vitro* translated proteins were incubated with or without muristerone A (Muri) and $[^{32}P]$ labeled probes (5 fmol, $\sim 10^4$ cpm) as indicated. Supershifts were performed with the addition of 1 µl AB11 to the binding reaction and are marked with the arrowhead. Lanes 1, 7, 13, and 19 represent incubation of DNA fragments with unprogramed reticulocyte lysate. Gels were run for 2.5 h (17c, 83c) and 3 h (32c, 42c), respectively.
Results

As there are no EcR isoforms known in *Chironomus* and as the two forms of cUSP are not different in their DNA-binding properties (Vögtli et al., 1999), we focused on the binding affinity of both cEcR and cUSP-2 (referred to here as cUSP) alone in the absence or presence of ligand (Fig. 17). The expression of the respective *in vitro* translated proteins was performed as before, using the pSCT-constructs. In accordance with previous findings we obtained no shifts with probe 57c (not shown). To our surprise, also probe 55c was not able to bind neither EcR nor USP in this experiment (not shown). For the remaining probes 17c, 32c, 42c, and 83c, we did not detect any binding of EcR alone with or without hormone. The faint band detected with probe 17c (lane 4) maybe suggests a weak binding of EcR alone, but this has to be verified. The binding property of USP alone to the *Chironomus* fragments is not as prominent as for the above described *Drosophila* fragments. As depicted in Figure 17, only probe 17c and 32c showed a shifted band with USP alone but only as supershifts with AB11 (lanes 6, 12; arrowhead). The same probes revealed no affinity to USP alone without antibody (lanes 5, 11). Thus, it seems that the binding pattern with three shifted bands seen in lane 2 for 17c and lane 8 for 32c, respectively, is not due to binding of either EcR alone or USP alone (see also Fig. 7D, lanes 2, 4). It rather indicates the binding of multimers to the respective fragments. In addition to the negative results concerning binding of EcR alone, probes 42c and 83c are also not able to bind to USP alone (lanes 15, 16, 17, 18, 21, 22, 23, 24).

In conclusion, these data demonstrate that binding of either EcR or USP alone to genomic elements is a possibility, at least in *Drosophila*, and that the ligand somehow defines the nature of this binding (monomeric or homodimeric binding) whereas the DNA fragment defines EcR isoform preferences. The impact of these findings on our understanding of ecdysone induced gene regulation will be discussed (see Discussion).
4. DISCUSSION

The identification of target sites for the EcR/USP complex as well as the corresponding genes is an important step towards elucidating the molecular mechanisms underlying ecdysone function during insect development. Here, we have demonstrated the feasibility of using specific antibodies and a DNA-binding assay to screen for EcR/USP binding sites in the genome of two different insect species, Chironomus tentans and Drosophila melanogaster. Genomic DNA fragments bound to bacterially expressed EcR and USP were immunoselected with a specific antibody raised against USP and the enriched DNA cloned after six rounds of whole genome PCR (for scheme see Fig. 3A). After confirmation by competition gel mobility shift assays using in vitro translated proteins and by sequence analysis, six unique binding sequences from C. tentans and four unique sequences from D. melanogaster were identified and further characterized by band shift assays, Southern analysis, and in situ hybridization to isolated polytene chromosomes (for summary of the results see Table 5).

Table 5 (next two pages). Main characteristics of fragments selected from the genomes of Drosophila melanogaster and Chironomus tentans, respectively

Main characteristics of the five unique fragments as well as the three subfragments of 7d for Drosophila are shown in Table 5A. The six unique fragments of Chironomus are shown in Table 5B. The fragment length, the localization of half-sites and the putative binding motif DR8/DR1 (see Fig. 5 and 6), a comparison to the database, the results of EMSA experiments (see Fig. 7, 13–17), Southern hybridization to genomic DNA (see Fig. 8) as well as in situ hybridization (ISH) to isolated chromosomes (see Fig. 10 and 11) are indicated in the table. The different marks are as follows: –, does not exist; /, not analyzed; + (competition EMSA), class II fragments according to Table 2 (i.e. partial competition); ++ (competition EMSA), class I fragments according to Table 2 (i.e. complete competition); + (direct EMSA), weak binding element; ++ (direct EMSA), strong binding element; + (Southern blot), confirmation of genomic origin. The mapping to chromosomal loci is indicated (see Table 3 and 4). For further explanation see the legends to the respective figures and tables or see text.
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Discussion

4.1 Whole genome PCR – a useful approach to select for genomic EcR/USP binding sites

Several methods for the isolation of target sequences of transcription factors have been reported. Selection via mobility shift assays (Shago and Giguère, 1996), microdissection of protein-bound chromosomal loci (Saunders et al., 1989), differential or subtractive hybridization (Manning et al., 1990; Pellerin et al., 1993), and filter binding assays (Inoue et al., 1991) proved to be successful procedures especially if no specific antibodies exist. However, they are somehow limited in that they need a high amount of proteins (e.g. filter binding assay) or that they are very time-consuming (e.g. microdissection). If antibodies are available, the most common and powerful method used for the selection of protein-bound DNA sequences is the immunoprecipitation via specific antibodies (Gould et al., 1990; Sompayrac and Danna, 1990; Bigler and Eisenman, 1994; Phelps and Dressler, 1996). This relatively simple and fast technique can enrich for DNA fragments that are bound by the protein(s) of interest either in vitro or in vivo. Furthermore, by performing multiple cycles of immunoprecipitations, binding sites can be purified from a genomic library essentially to homogeneity even if they are occurring only once in the genome (Sompayrac and Danna, 1990). To preserve the formed protein-DNA complexes from dissociation especially when working in vivo, some investigators used protein-crosslinked chromatin (Graba et al., 1992; Orlando and Paro, 1993; Tomotsune et al., 1993), which has the disadvantage of a poor cloning efficiency of the recovered DNA. A limitation of all selection methods mentioned above, however, is the enrichment of only high-affinity binding sites.

For our project, we decided to use a combination of immunoprecipitation and subsequent PCR-amplification to select for genomic EcR/USP binding sites. This so-called whole genome PCR approach has been employed previously to identify DNA-binding sites for the p53 tumor suppressor protein (El-Deiry et al., 1992), ER (Inoue et al., 1991), RAR (Costa-Giomi et al., 1992; Rudert and Gronemeyer, 1993), or TR (Caubin et al., 1994; Iglesias et al., 1996). A first test for the efficiency of the method using the selected cloned fragments in competition EMSA revealed a yield of less than
50% of clones that were able to compete for binding of EcR/USP to the synthetic PAL1 element (Table 2). This relatively low efficiency could be explained by the nature of this PCR-based selection approach: in addition to the amplification of selected DNA fragments specifically bound to EcR/USP also unspecifically bound DNA fragments are amplified with a very low binding affinity either to EcR/USP, to the antibody or to the magnetic beads. As expected, this latter fraction of amplified DNA does not show binding to EcR/USP in competition EMSA (Fig. 4).

4.2 Structure variability of putative EcREs from *Chironomus* and *Drosophila*

It was very surprising to us that none of the so far identified EcREs could be isolated with our whole genome PCR approach. However, there still exists the possibility that selected DNA fragments containing these binding elements have not yet been cloned or that we did not pick these clones for our first screening by competition EMSA. When comparing the nucleotide sequences of the presented fragments from either *Drosophila* or *Chironomus* to known EcREs we found single half-sites of *Drosophila* EcREs in all of our fragments (Fig. 5 and 6; Table 5). Clones 7d and 38d from *Drosophila* are the only fragments which harbor a complete DR8/DR1 motif composed of AGGTCA half-sites (Fig. 6). These findings evoke the fundamental question whether and how multiple single half-sites as well as the DR motif might contribute to EcR/USP binding. DR motifs composed of AGGTCA are known to be functional elements for many nuclear receptors, even in *Drosophila* where the EcRE of the *ng* gene is composed of such a DR (D’Avino et al., 1995). For ER, it has been demonstrated that DRs composed of multiple AGGTCA half-sites separated from each other more than 100 bp can act as a functional ERE in the chicken ovalbumin promoter region (Kato et al., 1992). Moreover, it has been shown that DRs spaced by 10 to 200 bp can function as HREs for the VDR and the retinoid acid receptors (Kato et al., 1995). Thus, one might speculate that gene regulation through the cooperative action of widely spaced half-sites also exists in insects.
To tackle the problem of putative EcREs in our fragments, we first focused on the DR motif present in 7d/38d. First evidence for the hypothesis that the DR8/DR1 is involved in EcR/USP binding were obtained by EMSA experiments using subfragments of 7d. Only subfragment 7d[160-307] containing the composed DR element revealed nearly the same affinity binding to EcR/USP as shown for fragment 7d (compare Fig. 13B and 14A). However, it seems that the flanking sequences represented by the other subfragments 7d[1-159] and 7d[308-538] are also involved in EcR/USP binding although they are very weak binding elements in EMSA (Fig. 16; Table 5A). To confirm these data and to determine the putative EcRE in clone 7d at the nucleotide level, we started performing DNaseI footprinting analysis using in vitro translated EcR/USP (pcDNA3-constructs) together with subfragment 7d[160-307]. After digestion with various concentrations of DNaseI, one strand of DNA was $^{32}$P labeled by primer extension using T7 or T3 as radiolabeled primer followed by separation on 6 % denaturing PAGE (Wellinger and Thoma, 1996). Unfortunately, the obtained DNA pattern did not yet allow us to distinguish between protein bound (i.e. a so-called footprint) and non-bound nucleotides (not shown). Probably the ratio of receptor proteins to DNA template has to be further optimized. Another possibility for the determination of nucleotides which form the putative EcRE would be the use of mobility shift assays with short subfragments containing the sequences of interest as competitors and as labeled probes. Site-directed mutagenesis would also help in defining nucleotides that are necessary for EcR/USP binding.

Besides the characterization of putative EcREs in our selected fragments, we also wanted to know whether the perfect PAL1 composed of two AGGTCA half-sites exists as a natural EcRE in C. tentans or D. melanogaster. Recently, this motif has been defined by EMSA experiments as being the best synthetic EcR/USP binding element (Vögili et al., 1998; Wang et al., 1998). To test its genomic occurrence, in situ hybridizations were performed using a biotin labeled 128 bp fragment containing one copy of the perfect PAL1 (5'-GAGGTCAGTGACCTC-3') for D. melanogaster and a 240 bp BamHI/BglII subfragment of pSP-PAL1/AT(8x) containing eight copies of
Discussion

PAL1 for *C. tentans*, respectively. In both species, no signals were detected, indicating that this element is not present in the genome (not shown). However, the resolution of *in situ* hybridization is rather low and it is possible that the fragments were too short for efficient labeling and/or that they failed to hybridize under the given conditions. So far, we can conclude that the results presented here support previous findings (see Introduction) that EcR/USP binding sites reflect an unusually high level of variability making consensus site calculation difficult.

4.3 Both, EcR and USP alone are able to bind to the selected DNA fragments

It is common knowledge that steroid hormone receptors like ER, GR, or PR bind to their response element exclusively as homodimers (Beato et al., 1995). Curiously, the insect steroid receptor EcR is an exception in that it is thought to be ecdysone-inducible and functional only as a heterodimer together with USP, the insect homolog of the vertebrate RXR. Thus, the ecdysone receptor more closely resembles the vertebrate family of RXR heterodimeric receptors, rather than the family of vertebrate steroid receptors. All three EcR isoforms found in *Drosophila* (EcR-A, EcR-B1, EcR-B2) are equal in their binding affinity to USP (Yao et al., 1992; Yao et al., 1993; Talbot et al., 1993; Thomas et al., 1993). In contrast to the EcR/USP complex, additional heterodimers formed by both EcR and USP with other orphan receptors are not ecdysone-inducible (Sutherland et al., 1995; Zelhof et al., 1995a; White et al., 1997; Crispi et al., 1998). By using *in vitro* translated EcR from two different insect species (*D. melanogaster, Bombyx mori*) several authors failed to demonstrate binding of EcR alone either to the *hsp27* EcRE or to synthetic elements (PAL1, DRs) when tested in EMSA (Yao et al., 1992; Yao et al., 1993; Thomas et al., 1993; Horner et al., 1995; Antoniewski et al., 1996; Swevers et al., 1996; Vögtli et al., 1998). This is in clear contrast to the data presented here and in another study (J.-F. Mouillet, unpublished) showing for the first time DNA binding of *in vitro* translated *Drosophila* EcR isoforms in the absence of the heterodimer-partner USP: in our EMSA experiments we could show binding of all three *Drosophila* EcR isoforms alone to a synthetic element.
(PAL1), to the hsp27 EcRE (J.-F. Mouillet, unpublished results) as well as to the selected fragments 7d, 38d, 32d and subfragment 7d[160-307]. How can we explain these contradictory results? We noticed that the use of pcDNA3 as expression vector for the synthesis of in vitro translated proteins led to high expression levels compared to the pSCT vectors (Fig. 12) or vectors like pBluescript or pCMX used in the studies mentioned above, and that this was needed to visualize this kind of binding in EMSA experiments. In addition, other groups carried out their studies only with EcR-B1, about which we have little information from our own experiments due to the low expression level of this isoform (Fig. 12A, lanes 2, 4). With fragments like 42d, which only show weak affinity binding even to the heterodimeric EcR/USP complex, we were not able to show binding to EcR alone. Thus, binding of the three EcR isoforms in the absence of USP - or, at least, the detection of this binding - seems to be dependent on the expression level of the proteins and on the affinity of a DNA sequence to EcR.

Binding to USP alone has been shown previously for some DR elements (D'Avino et al., 1995; Antoniewski et al., 1996; Vögtli et al., 1998; Wang et al., 1998) as well as for PAL1 (Wang et al., 1998) when performing mobility shift assays using in vitro translated receptor. These findings could be confirmed by our EMSA experiments at least for some of the selected fragments, i.e. the Drosophila fragments 7d, 38d, 32d and the subfragment 7d[160-307] (Table 5A). Both monomeric and homodimeric dUSP binding, i.e. two shifted bands, could be found only when using 7d/38d, but neither with 32d nor with the subfragment 7d[160-307] (Fig. 13 and 14). It seems that the DR motif is a necessary part of the putative USP binding motif but that some flanking regions, which are located either up- or downstream of 7d[160-307] are also needed for cooperative binding of two dUSP molecules. It would be interesting to test whether and how USP binding is influenced by mutations in the DR motif or mutations in the single half-sites found in all fragments. Concerning the Drosophila fragment 42d and the other two subfragments of 7d, it seems that the capability to bind to the heterodimer with high affinity is a prerequisite for binding of USP (Fig. 15 and 16) as already seen for binding of EcR (see above).
The results obtained with the selected fragments from *C. tentans* are not so easy to interpret. At first sight it seems that there exist species-specific differences in binding of either EcR or USP alone to DNA elements: with none of the selected *Chironomus* fragments we obtained shifts when incubated with EcR alone, independently of the addition of muristerone A (Fig. 17). The faint band that is visible with probe 17c in the absence of ligand (lane 4) has to be verified with supershift experiments. In contrast to *Drosophila* (see above), in *Chironomus* there exists some precedent evidence for binding of cEcR alone: using bacterially expressed and purified *Chironomus* EcR, Elke et al. (1997) could show in EMSA experiments binding of a cEcR homodimer to the synthetic PAL1. However, it should be noted that these experiments were performed in the presence of detergent (0.03 % sarkosyl) and BSA (20 mg/ml). Although bacterially expressed and purified EcR/USP proteins from both *C. tentans* and *D. melanogaster* were used for our selection approach, the findings of Elke et al. do not seem to play such an important role in our system due to the selection via the anti-USP antibody AB11. Consequently, our selected fragments should be definitely recognized by USP either as monomer, homo- or heterodimer since EcR has no affinity to AB11. Nevertheless, this result provide further indications that binding of EcR alone might be a possibility which one has to consider.

Concerning our EMSA experiments with cUSP alone, we detected a clear shift with two of our fragments (17c, 32c) but only as supershifts (Fig. 17, lanes 6, 12), indicating that this kind of binding can occur also in *Chironomus*. The fact that this binding is only visible after the addition of the specific anti-USP antibody AB11 suggests a very weak and/or unstable binding of USP to these two *Chironomus* fragments. One problem for the detection of binding of EcR and USP alone in *C. tentans* could be the use of the pSCT-constructs for the in vitro translation reactions. It would be interesting to repeat these EMSA experiments with pcDNA3-constructs and to compare the expression levels of the different constructs, which were not the same for the *Drosophila* receptor proteins (Fig. 12). Another, rather simple explanation for the observed differences between *Chironomus* and *Drosophila* might be that binding of EcR or USP alone
strongly depends on the structure of the DNA element independently of the insect species.

Whether the above reported binding properties of both in vitro translated EcR and USP in the absence of their partner reflect binding of mono- and/or homodimeric EcR and USP, respectively, rather than DNA binding of newly formed heterodimers with factors present in the reticulocyte lysate will be discussed below.

4.4 The A/B domain of Drosophila EcR plays a role in DNA binding

The three Drosophila EcR isoforms are encoded by only one gene, which is in contrast to other receptor isoforms including the four RXR isoforms encoded by three genes, the TR isoforms encoded by two different genes, and the eight RAR isoforms encoded by three genes (Giguère, 1994; Refetoff et al., 1994; Kastner et al., 1995). The EcR isoforms are expressed in a stage- and tissue-specific manner, with the A isoform predominantly expressed in adult tissues and the B1 isoform predominantly expressed in larval tissues (Talbot et al., 1993). They only differ in their N-terminal A/B domain and contain the same DBD and LBD (Talbot et al., 1993). It has been suggested that tissue-specific coactivators are responsible for the determination of which EcR isoform is used to activate a gene in different tissues. As expected, EMSA studies using all three possible EcR/USP heterodimer combinations revealed equal DNA-binding affinities independently of the EcR isoform (Koelle, 1992; Talbot et al., 1993) which we could confirm using our selected fragments. Unexpectedly, similar experiments performed in the absence of USP showed isoform-specific DNA-binding properties depending on the DNA fragment we used: probe 7d preferably was bound by the B1 isoform rather than by EcR-A. With 38d, 32d, and 7d[160-307] more pronounced shifts were obtained with EcR-A than with EcR-B2 (Table 5A). Thus, it seems that EcR isoform-specific sequences located in the A/B domain can somehow influence the DNA-binding properties but that these differences are detectable only if the heterodimerization partner USP is missing.
The N-terminal A/B region of nuclear receptors is diverse in size and amino acid composition, which suggests that it may play a role in conferring cell type and/or promoter specificity. That this domain affects the DNA-binding properties in addition to its role in transcriptional activation and binding of factors like TFIIB or coactivators has been reported for several other members of the nuclear receptor superfamily, including TR, ROR, and AR. Among the known TR isoforms, which differ primarily in their A/B domain, TRβ1 and TRβ2 bind to a palindromic TRE predominantly as homodimers, whereas TRα prefers binding to the same element as a monomer (Darling et al., 1993; Hollenberg et al., 1995). A unique motif of five basic aa which is only present in the A/B domain of TRα has been identified as being responsible for the specific DNA-binding properties of this isoform (Hadzic et al., 1995; Hadzic et al., 1998). The importance of N-terminal sequences for the DNA recognition by different TR isoforms could be further confirmed by comparing the DNA-binding properties of the oncogenic isoform v-erb A with the non-oncogenic TRα (Chen et al., 1993; Judelson and Privalsky, 1996). These studies revealed that the DNA-binding specificity of these two isoforms is the product of the action of two different amino acids in the zinc finger domain in conjunction with two different amino acids located in the A/B domain. Intriguingly, the effects of the N-terminal amino acids on DNA recognition were manifested only if the amino acids in the DBD were compatible, i.e., if both the DBD and the A/B domain derived from the same isoform (Chen et al., 1993). Similar results were obtained with the different splice variants of the orphan receptor RORα. Here, the N-terminal sequences of RORα2 have an inhibitory influence on DNA binding, which results in binding to DNA elements with strict specificity. On the contrary, the A/B domain of RORα1 is needed for optimal DNA binding of this isoform to a variety of response elements (Giguère et al., 1994). Recently, it has been reported for AR that sequences in the A/B domain play an important role in the stabilization of DNA binding (Gast et al., 1998). Deleting the entire A/B domain led to a mutant AR that had lost almost all of its DNA-binding function. However, these effects do not seem to be sequence-specific.
Discussion

Several models exist trying to explain how the N-terminal sequences of nuclear receptors might be capable of such effects. Conceivably, the interaction may represent the form of a direct physical contact of amino acids of the A/B domain with the DNA-binding element. However, it is doubtful that such direct contact exists because of spatial constraints. As an alternative, amino acids of the A/B domain could interact elsewhere on the DNA to bend the response element in order to facilitate binding. This also does not seem to be very likely; it could be demonstrated that bending of DNA through TRα and v-erb A is the same and hence independent of the nature of the N-terminus (J. Hamaguchi and M. L. Privalsky, unpublished data). Another hypothesis is that intramolecular interactions occur in which the N-terminal amino acids would modify the conformation of the DBD by influencing the tertiary structure. This would then lead to an alteration of the precise position or orientation of the DBD and thus to a different half-site recognition. It is known that intramolecular interactions via coactivators acting as bridging proteins between the N-terminal AF-1 and the C-terminal AF-2 are necessary for the full transactivation function of nuclear receptors (Nagpal et al., 1992; Kraus et al., 1995; Ouate et al., 1998). Possibly, this might influence the DNA-binding properties through changing of the overall conformational structure of the receptor. Since the recruitment of coactivators to the AF-2 located in the LBD is ligand-dependent, binding of the ligand might indirectly influence the DNA-binding properties of receptors (see next chapter). That such interactions of accessory proteins with the A/B domain of nuclear receptors may account for the above reported isoform-specific DNA-binding properties is not very likely for the following reasons: the above mentioned in vitro DNA-binding studies with TR and RORα were performed with either proteins expressed in E. coli or receptors synthesized as in vitro translated proteins using rabbit reticulocyte lysates, as they were in our case. Nevertheless, concerning the in vitro translated receptors, we cannot totally exclude possible interactions of the receptors A/B domain with still unknown isoform-specific factors present in the lysate. Since the studies with AR were carried out using transiently transfected COS-7 cells as protein source (Gast et al., 1998), here it is conceivable that the effects of N-terminal sequences on DNA binding may arise from the recruitment of
Discussion

additional proteins present in the cell extracts. Definitive answers showing direct interactions of the A/B domain with the DNA element or with the DBD ultimately require crystallographic analyses which so far were not performed in the presence of the N-terminal domain of nuclear receptors.

4.5 The DNA-binding properties of Drosophila EcR are changed by ligand

As already mentioned above, binding of the ligand causes conformational changes in nuclear receptors which lead to a release of corepressors, recruiting of coactivators, and finally, the activation of the target gene. Since both ligand binding and dimerization co-localize to the C-terminal E domain, it is conceivable that a key control of ligand binding is the stabilization of dimeric receptor complexes. Our EMSA experiments revealed that apart from the above discussed isoform-specific differences concerning DNA binding of EcR in the absence of USP, the effect of ligand on this kind of binding was relatively independent of the used isoform (Fig 13 and 14): homodimeric binding of either EcR-A, EcR-B1 or EcR-B2 to 7d, 38d, 7d[160-307] and of EcR-A to 32d, respectively, was almost completely abolished after the addition of muristerone A. Furthermore, in experiments with EcR-A or EcR-B1 together with 7d, 38d, and 32d low molecular weight shifts could be detected in the presence of ligand indicating binding of monomers (Fig. 13 and 14B). The fact that the ligand can alter the dimerization status of DNA-bound nuclear receptors has been shown previously for other ligands, such as thyroid hormone, vitamin D3, and 9cRA. For VDR, it has been demonstrated that the formation of DNA-bound homodimers occurs through a monomeric intermediate and that DNA binding is mandatory for the formation of homodimers in the absence of ligand (Cheskis and Freedman, 1994). In their model it has been proposed that binding of the ligand causes (i) enhanced dissociation of DNA-bound homodimers and (ii) at the same time a decreased rate of conversion of DNA-bound monomers to homodimers. With TR, similar effects could be observed, although here both DNA-bound monomers and homodimers were found in the absence of ligand, depending on the sequence of the DNA element (Ribeiro et al., 1992; Yen et al., 1992; Miyamoto et al., 1993).
thyroid hormone, however, TR revealed exclusively monomeric binding. Somewhat altered binding properties were reported for RXR which is able to form tetramers with a high affinity (Kersten et al., 1995a; Kersten et al., 1995b; Kersten et al., 1997). Upon ligand binding these higher order complexes dissociate to DNA-bound RXR homodimers. For all these examples it has been proposed that homodimerization/tetramerization of receptors may function in silencing or inactivation of the respective receptors. In contrast to TR or VDR, which are poorly transcriptionally active as homodimers, inactivation of RXR requires the sequestration of both monomers and homodimers, a need that can only be fulfilled by the formation of tetrameric RXR complexes which are not able to transactivate (Kersten et al., 1997). It has been suggested that the ligand-induced formation of VDR or TR monomers is necessary for subsequent heterodimerization with RXR since both seem to function predominantly via heterodimers with RXR. From our results it might be speculated that gene transcription mediated through EcR is regulated in a similar way. Thus, the detected monomeric DNA binding of EcR in the presence of muristerone A could be explained as an intermediate step required for the formation of a transcriptionally active EcR/USP heterodimer. The lack of monomeric binding of EcR to probe 7d[160-307] in the presence of ligand could be explained by either a very low affinity or instability of such an EcR monomer bound to this DNA fragment. The opposite seems to be the case with fragments 7d, 38d, and 32d with which monomeric binding could be detected in the presence of hormone (Fig. 13 and 14). It would be interesting to know whether EcR homodimers or monomers, alone or in association with other factors, can act as hormone-dependent repressors or activators of transcription when bound to different classes of EcREs such as our selected fragments.

So far, the order of events in EcR mediated gene activation is still speculative. It is not known whether the first step is DNA binding, as proposed for non-steroid receptors, ligand binding, as proposed for steroid receptors like GR, or heterodimerization with USP. From our EMSA studies using in vitro translated EcR and USP we know that DNA binding and heterodimerization occur even in the absence of muristerone A (not
Discussion

shown). Furthermore, as shown in vivo for both C. tentans (Wegmann, 1994) and D. melanogaster (Koelle et al., 1991), EcR is predominantly located in the nucleus even in the absence of ligand. These two findings would argue for binding to DNA even in the absence of hormone. Concerning the formation of heterodimers, it has been demonstrated by a yeast two-hybrid assay that the E domains of both dEcR and dUSP are able to interact in the absence of a DNA element and that this interaction is enhanced by the addition of hormone (T. Bergman, personal communication). However, homodimerization of two dEcR E domains could not be detected in the yeast system, indicating either the involvement of other receptor domains or the need of DNA binding for this kind of interaction as proposed for VDR (see above). The observed ligand effects on EcR binding to DNA could be explained by conformational changes on EcR caused by ligand binding. This could inhibit the formation of EcR homodimers and/or the binding of ligand-bound EcR monomers to the DNA element due to sterical hindrance. Thus, only DNA-bound monomers are identified which would be able to form transcriptionally active EcR/USP heterodimers. Since we used in vitro translated EcR as source for our DNA binding studies, we cannot rule out that these effects are due to the association of EcR with proteins present in the reticulocyte lysate which was used for in vitro translation reactions (see above). Such a protein might be RXR, which could form a heterodimeric complex with EcR capable of binding to our selected fragments. Thus, the band shifts obtained with EcR alone in the presence of hormone might be misinterpreted as monomeric binding and could also be the result of DNA-bound EcR/RXR heterodimers. Evidence for this hypothesis could be provided by a comparison of the migration properties of this shifted band with the shift resulting from USP homodimeric binding; both shifted bands should approximately have the same velocity with a slightly faster migration of a DNA-bound EcR monomer. This, however, is not the case. Moreover, the shifts obtained with EcR alone in the absence of ligand might also represent a DNA-bound receptor complex of a higher order than a homodimer. However, an exact determination of the molecular weight based on the migration properties of the identified complexes is very difficult since these in vitro binding reactions are separated on native gels. Additional experimental evidence is
required to investigate this problem and to confirm the ligand-effect on EcR bound to DNA. Whether these findings can be extrapolated to the far more complex in vivo situation remains to be shown.

Another aspect of the experiments performed by Cheskis and Freedman (1994) showing the influence of different ligands on DNA-bound VDR/RXR heterodimers might be important with respect to gene regulation triggered by the ecdysone receptor complex. While the formation of VDR/RXR bound to DNA was promoted and stabilized through VDR-specific ligands, the addition of 9cRA resulted in the dissociation of this heterodimeric complex. Consequently, RXR would then be able to act in another signaling pathway either as RXR homodimer or together with other nuclear receptors. Although these results were obtained by in vitro experiments, this mechanism could also reflect the in vivo situation of hormone-regulated gene transcription. Moreover, this model could be also applicable to gene regulation mediated through the EcR/USP complex under the following two conditions: first, let us assume that ecdysone and its agonist muristerone A have the same capability of transforming DNA-bound EcR homodimers into DNA-bound monomers as does a VDR-specific ligand in the case of VDR. Second, we have to accept the postulate that juvenile hormone acts either indirectly or directly on USP (Jones and Sharp, 1997; Hall and Thummel, 1998) akin to the effect of 9cRA on RXR. It is then tempting to speculate that fluctuations in the concentration of both hormones could regulate insect development via stabilization or destabilization of an EcR/USP heterodimer (Lezzi et al., 1999). Thus, the decreased concentration of a putative USP ligand at the end of larval development together with a rise in ecdysone titer would lead to a favored gene regulation mediated through the action of the EcR/USP complex.
4.6 What are the functions and the corresponding genes of our putative EcREs?

Although the above question cannot be answered by the present work, we can provide some hints concerning the possible role of our selected fragments in ecdysone-triggered gene regulation. These indications result from data obtained with in situ hybridization to polytene chromosomes (Fig. 9, 10, and 11; Table 5). Several selected fragments of both insect species hybridized to characterized sites which are known to be regulated by ecdysone. However, we have to consider the resolution of approximately 100 kb per stained band obtained by this technique making an identification of the cytological loci rather difficult.

For C. tentans, these stained sites include the loci II-14A, IV-2B, IV-5C, I-20A, II-19A, and II-19B (Fig. 9 and 10; Table 3). Unfortunately, the first three of the above mentioned loci were only stained with the selected but uncloned DNA mixture and could not be recovered by the individually cloned fragments. Nevertheless, these three loci are very interesting: whereas locus II-14A which harbors the cUSP gene (Wegmann et al., 1995) forms a late ecdysone-regulated puff (Clever, 1961), locus IV-2B forms an early ecdysone-inducible puff and encodes the Chironomus E75 (cE75) (Clever, 1961; Clever, 1964; Clever, 1966; Wegmann et al., 1995). This is in agreement with the Drosophila E75 gene which localizes to the early puff 75B (Feigl et al., 1989; Segraves and Hogness, 1990). The other stained locus on chromosome IV (IV-5C) forms a so-called HS puff, meaning decoration with endogenous EcR only after incubation for 1 h at 39°C (Lezzi, 1996). The identified loci obtained with the cloned fragments 42c (loci I-20A and II-19B2), 55c (locus II-19B) and 57c (loci II-19A and II-19B2) also belong to this class of puffs induced by heat shock. With the exception of puff-site I-20A, which is stained by antibodies against cEcR even at physiological temperatures (Wegmann et al., 1995), intense staining of loci II-19A, II-19B as well as IV-5C with an anti-cEcR antibody is only detected after heat shock. In addition to the decoration with endogenous EcR, all identified HS puffs are also decorated with HSP and heat shock
factor (HSF) upon heat shock (Lezzi, 1996). Concerning the double bands II-19A and II-19B stained with probe 57c, it should be noted that this pattern has been also observed with both anti-cEcR and anti-HSP antibodies after heat shock. The pattern obtained by probe 42c, with two stained loci on two different chromosomes, could be explained by repetitive sequences, which conforms with the results obtained by Southern blot analysis (Fig. 8B, lanes 4, 5). Thus, in situ hybridization with the selected fragments 42c, 55c, and 57c provides some evidence that these sequences might be involved in ecdysone regulation and that heat shock might play a certain role. One of the next steps would be the successful isolation of the fragments corresponding to stained chromosomal loci II-14A, IV-2B, and IV-5C.

The two hybridization signals obtained by the *Drosophila* fragment 7d which map to chromosomal loci X-12E and X-13B correspond to known puff-sites (Ashburner, 1972b; Ashburner, 1975a; White et al., 1997). The assumption that these puff-sites are involved in the ecdysone response network finds further support in immunological studies using antibodies against various members of the nuclear receptor superfamily and against the early gene product E74A: whereas Lavorgna et al. (1993) could show binding of FTZ-F1 only to 13B but not to 12E, this staining pattern is reversed with anti-E74A which only binds to 12E (Urness and Thummel, 1990). Both loci are stained by anti-DHR3 (Lam et al., 1997; White et al., 1997) and anti-DHR78 (Fisk and Thummel, 1998). Interestingly, these findings possibly could provide some more evidence for our hypothesis that the DR motif and/or single half-sites identified in fragment 7d/38d are indeed recognized by nuclear receptors in vivo: binding to direct repeated DNA motifs composed of two AGGTCA half-sites as well as to the distal element of *Eip28/29* has been reported previously for DHR78 (Fisk and Thummel, 1995; Zelhof et al., 1995b). For βFTZ-F1 binding to the ng EcRE which is composed of two AGGTCA half-sites arranged as direct repeat could be shown (Crispi et al., 1998), whereas DHR3 prefers monomeric binding to single AGGTCA half-sites preceded by an AT-rich sequence (Horner et al., 1995). Thus, it would be interesting to test whether 7d/38d as well as subfragments of 7d could bind to the above mentioned receptors. The
use of DNA fragments containing mutations in the DR motif and/or the single half-sites together with the various receptors should help in unraveling the role of these sequences for receptor binding.

Although the locus 41F stained by the *Drosophila* fragment 32d could not be correlated to a known puff-site, the vicinity to locus 42A encoding EcR invites speculations about a possible function as regulatory sequence for the *EcR* gene. First, locus 42A forms an ecdysone-inducible puff (Ashburner, 1972a; Ashburner and Richards, 1976). Second, the peaks in the expression pattern of EcR correspond to peaks in the ecdysone titer which led to the suggestion that EcR is induced by ecdysone (Koelle et al., 1991). In this model, ecdysone would stimulate the expression of its own receptor in analogy to estrogen, retinoic acid or vitamin D3 and the genes encoding their respective receptors (Barton and Shapiro, 1988; de The et al., 1989; Mangelsdorf et al., 1987). Thus, a correlation to the ecdysone regulation of the *EcR* gene should be considered as one possible function of element 32d.

4.7 Perspectives

The results presented here are only based on *in vitro* experiments. However, some of our findings implicate a correlation with the *in vivo* situation (see above). For the future, two important aspects should be elucidated by experimental data: first, the characterization of the respective genes regulated through our selected EcR/USP binding sites and second, the physiological functionality of these putative EcREs. Considering both the length of our selected fragments varying from 228 to 538 nt and the fact that the majority of the so far characterized *Drosophila* EcREs are located at most 753 nt upstream of the transcription initiation site of their respective target gene (see Introduction), hybridization of our selected fragments to cDNA libraries might be a possibility to identify corresponding genes. The feasibility of such an approach has already successfully demonstrated for the identification of TR regulated genes (Caubin et al., 1994). In order to investigate possible functions of the presented EcR/USP
binding sites, transient transfection assays should be performed. These experiments will provide evidence whether these newly identified binding sites act as positive (i.e. induction of a target gene) or negative (i.e. repression of a target gene) regulatory elements in the presence of ecdysone. Finally, the use of transgenic flies carrying these binding sites as GFP fusion proteins will ultimately elucidate their role in the natural environment.

In the Introduction, we mentioned the well characterized genetics as one advantage when working with *Drosophila*. Sequencing of the whole genome of *Drosophila melanogaster* is currently underway and should be finished by the end of 2001 (statement of the Berkeley *Drosophila* Genome Project). The results of this project will certainly lead to a more detailed knowledge about our selected fragments.
5. APPENDIX

**Abbreviations**

- **aa**: amino acid(s)
- **AF-1**: activation function 1
- **AF-2**: activation function 2
- **AR**: androgen receptor
- **CBP**: CREB binding protein
- **cDNA**: complementary DNA
- **COUP-TF**: chicken ovalbumin upstream promoter transcription factor
- **cpm**: counts per minute
- **9cRA**: 9-cis retinoic acid
- **DBD**: DNA-binding domain
- **DNA**: deoxyribonucleic acid
- **DR**: direct repeat
- **ds**: double stranded
- **EcR**: ecdysone receptor
- **EcRE**: ecdysone response element
- **ER**: estrogen receptor
- **FISH**: fluorescence in situ hybridization
- **FXR**: farnesoid X receptor
- **GCNF**: germ cell nuclear factor
- **GR**: glucocorticoid receptor
- **GRE**: glucocorticoid hormone response element
- **h**: hour(s)
- **HNF-4**: hepatocyte nuclear receptor 4
- **HRE**: hormone response element
- **HS**: heat shock
- **HSP**: heat shock protein
### Appendix

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>JH</td>
<td>juvenile hormone</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>kD</td>
<td>kilo Dalton</td>
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<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
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<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>min</td>
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<td>MR</td>
<td>mineralocorticoid receptor</td>
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<tr>
<td>N-CoR</td>
<td>nuclear receptor corepressor</td>
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<tr>
<td>NGFI-B</td>
<td>nerve growth factor-induced receptor B</td>
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<tr>
<td>nt</td>
<td>nucleotide(s)</td>
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<td>PAL</td>
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<tr>
<td>PCAF</td>
<td>p300/CBP-associated factor</td>
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<tr>
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<td>peroxisome proliferator-activated receptor</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>ROR</td>
<td>retinoid orphan receptor</td>
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<td>rpm</td>
<td>rounds per minute</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>sec</td>
<td>second(s)</td>
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<tr>
<td>SF-1</td>
<td>steroidogenic factor 1</td>
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<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid receptors</td>
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6. LITERATURE


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7. CURRICULUM VITAE

Personal details

Name
Nadja Katharina Seibel

Date of birth
April 22, 1967

Place of birth
Trier/Germany

Nationality
German

Education

1977 – 1986
Friedrich-Wilhelm-Gymnasium (secondary school), Trier
Abitur (A-levels): Grade 2.7 ("good")

1986 – 1987
Undergraduate studies in geography at the University of Trier

1987 – 1989
Language studies at the University of Trier:
Italian, Arabic, Russian

1989 – 1995
Undergraduate studies in biology at the Bayerische Julius-
Maximilian-Universität in Würzburg/Germany
Diploma thesis: Charakterisierung eines klonierten
Puppenkutikularprotein-Gens bei Galleria mellonella L.
(Grade: "very good")
Degree: Diplom Biologin (Grade: "good")

1995 – 1999
Graduate studies at the Swiss Federal Institute of Technology,
Institute for Cell Biology in Zürich/Switzerland
Ph. D. thesis: Identification and Characterization of Naturally
Occurring Ecdysone Response Elements in Chironomus
tentans and Drosophila melanogaster. Advisor: Prof. Dr. M.
Lezzi
Participation in Scientific Meetings

May 1997  I. Departement-Biologie-Symposium of ETH Zürich in Davos/Switzerland: poster presentation

September 1997  VIII. International Balbiani Ring Workshop in Lund/Sweden: oral presentation

March 1998  Keystone Symposium on Nuclear Receptor Gene Family in Lake Tahoe/USA: poster presentation

May 1998  II. Departement-Biologie-Symposium of ETH Zürich in Davos/Switzerland: poster presentation

July 1998  XIIIth Ecdysone Workshop in Jena/Germany: poster presentation