Doctoral Thesis

Mechanism for cAMP-induced, tissue-specific regulation of the urokinase-type plasminogen activator gene in kidney epithelial cells

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Publication Date:
1996

Permanent Link:
https://doi.org/10.3929/ethz-a-001710103

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Mechanism for cAMP-Induced, Tissue-Specific Regulation of the Urokinase-Type Plasminogen Activator Gene in Kidney Epithelial Cells

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of

Doctor of Natural Sciences

presented by

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1996
To My Parents
Wir haben das körperliche Indien
gesucht und Amerika gefunden; wir
suchen jetzt das geistige Indien -
Was werden wir finden?

H. Heine
1. SUMMARY ......................................................................................... 1

2. ZUSAMMENFASSUNG ..................................................................... 3

3. GENERAL INTRODUCTION ............................................................ 5
   3.1 Prologue .................................................................................. 5

3.2 Gene Expression is Regulated on Different Levels ...................... 6
   3.2.1 mRNA Stability is Regulated by Destabilizing Elements ......... 6
       3.2.1.1 The 5' Cap Structure ..................................................... 7
       3.2.1.2 The Destabilizing Elements Located in the 5' UTR ..... 8
       3.2.1.3 Destabilizing Elements in the Coding Sequence ......... 8
       3.2.1.4 The Destabilizing Elements in the 3' UTR ................. 9
       3.2.1.5 The Poly(A) Tail .......................................................... 11
       3.2.1.6 Trans-Acting Factors Involved in mRNA Decay ....... 11
       3.2.1.7 Possible Mechanisms for mRNA Decay ................. 12

3.3 Regulation of Eukaryotic Gene Transcription by cAMP ............. 14
   3.3.1 The Nuclear Target of PKA .................................................. 14
       3.3.1.1 The CREB/ATF-Family .............................................. 14
       3.3.1.2 The Basic Leucine Zipper .......................................... 16
       3.3.1.3 Structure and Function of CREB ............................... 16
       3.3.1.4 Structure and Function of ATF1 and CREM .......... 18
       3.3.1.5 Isoforms of CREB, CREMs and ATF1 .................... 20
       3.3.1.6 Interaction between CREB and the Basal Transcription Factors 20
   3.3.2 Role of CBP in the cAMP-Signal Transduction Pathway ....... 21
       3.3.2.1 Role of CBP in other Signaling Transduction Pathways 22
       3.3.2.2 p300 is Closely Related to CBP ................................. 24
   3.3.3 The cAMP-Response Element ............................................ 24
       3.3.3.1 The Consensus CRE and the CRE-Like Site ............ 25
       3.3.4 Mechanism of CREB-Dependent Gene Activation .......... 25

3.4 Characteristics of the Transcription Factor LFB3 ...................... 27
   3.4.1 Homeodomain Proteins: Structure and Mechanism of Transactivation, 27
       3.4.1.1 The Structure of the Homeodomain .......................... 27
       3.4.1.2 The POU-specific Domain ...................................... 28
       3.4.1.3 Transactivation by Homeodomain Proteins ............ 28
   3.4.2 The Homeodomain Family of LFB1 .................................... 29
       3.4.2.1 Introduction to LFB1/3, Evolution and Function ....... 29
       3.4.2.2 The Structure of LFB1/3 .......................................... 31
       3.4.2.3 DcoH, a Cofactor of LFB1/3 ................................. 34
       3.4.2.4 Distribution and Developmental Expression of LFB1/3 35
   3.4.3 Liver-specific Gene Expression .......................................... 36
       3.4.3.1 The cis-and trans-acting Factors ......................... 36
       3.4.3.2 A Network of Factors Coordinates the Development of Differentiated Hepatocytes 37

3.5 The Plasminogen Activators ..................................................... 39
   3.5.1 The Components of the Plasminogen Activation System ....... 39
       3.5.1.1 Plasminogen and Plasmin ....................................... 39
       3.5.1.2 The Plasminogen Activators .................................... 40
       3.5.1.3 Plasminogen Activator Inhibitors ............................. 41
   3.5.2 Assembly of the Plasminogen System on Cell Surfaces ........ 43
       3.5.2.1 Activation of the Plasminogen System on Cell Surfaces 43
       3.5.2.2 Plasminogen Receptors and uPA-Receptors ............ 43

3.6 Regulation of uPA Gene Expression in LLC-PK1 Cells ............... 45
   3.6.1 The LLC-PK1-Cells as a Model System for Studying Transcriptional Activation by the cAMP-Dependent Protein Kinase Pathway 45
3.6.2 cAMP-Dependent uPA Gene Expression in LLC-PK₁ Cells ........................................ 46
3.6.2.1 The ABC-Enhancer ................................................................................................. 47
3.6.2.2 Other Potential cAMP-Response Elements in uPA-Promoters of Various Species ...... 50

4. MATERIALS AND METHODS ................................................................. 52
4.1 Reagents ................................................................................................................. 52
4.2 Cell Culture ............................................................................................................ 53
4.3 Expression Vectors ................................................................................................. 53
4.4 Plasmids and Probes ............................................................................................. 54
4.5 Transient Transfection Assays ................................................................................ 54
4.6 RNA Isolation and Northern Blot Analysis ............................................................... 54
4.7 Determination of mRNA Stability ........................................................................... 55
4.8 Nuclear Transcription ............................................................................................. 55
4.9 Nuclear Extracts and Electrophoretic Mobility Shift Assays ...................................... 56
4.10 Protein Kinase A Treatment of Nuclear Extracts ...................................................... 57

5. RESULTS ............................................................................................................. 58
5.1 Role of LFB3 in Cell-Specific cAMP Induction of the Urokinase-Type Plasminogen Activator Gene .................................................................................................................. 58
   5.1.1 Summary ............................................................................................................. 58
   5.1.2 Introduction ........................................................................................................ 58
   5.1.3 Results ............................................................................................................... 60
       5.1.3.1 Cooperation of LFB3 in a cAMP-Responsive Enhancer ................................. 60
       5.1.3.2 Effect of cAMP and other uPA Inducers on LFB3 mRNA Levels ................. 61
       5.1.3.3 Effect of Br-cAMP and TPA on Domain C-Binding Activity ....................... 62
       5.1.3.4 No Change in Transcription Rate of the LFB3 Gene .................................. 65
       5.1.3.5 Induced Instability of LFB3 mRNA .............................................................. 66
       5.1.3.6 Effect of the Decrease in DNA Binding Activity of LFB3 on cAMP-Induction .... 67
   5.1.4 Discussion ......................................................................................................... 68
5.2 A CBP-independent Mechanism for cAMP Induction of the uPA Gene in LLC-PK₁ Cells .......................................................................................................................... 72
   5.2.1 Summary ........................................................................................................... 72
   5.2.2 Introduction ...................................................................................................... 72
   5.2.3 Results .............................................................................................................. 74
       5.2.3.1 Cooperation among three Domains in a cAMP-responsive Enhancer .......... 74
       5.2.3.2 Coupling of Hormonal and Tissue-specific Gene Regulation ....................... 75
       5.2.3.3 The ABC-Enhancer does not Mediate TPA-Dependent Gene Induction ......... 76
       5.2.3.4 ATF1 Interacts with the AB-Domains .......................................................... 77
       5.2.3.5 Binding of ATF1 and CREB to Domains A and B is Regulated by PKA-mediated Phosphorylation ............................................................... 80
       5.2.3.6 The Transactivation Domain of LFB3 is Required for ABC-Enhancer mediated cAMP-Induction ............................................................... 83
       5.2.3.7 A CBP-Independent Mechanism for the ABC-Enhancer mediated cAMP-Induction ............................................................... 85
       5.2.3.8 12S E1A has no Influence on the ABC-Enhancer ...................................... 85
   5.2.4 Discussion ......................................................................................................... 86
### 5.3 The ABC-Enhancer is Regulated Differently in F9 Cells than in LLC-PK₁

<table>
<thead>
<tr>
<th>Sections</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.1 Comparison between LLC-PK₁ and F9 cells</td>
<td>92</td>
</tr>
<tr>
<td>5.3.2 The difference between two ABC-Constructs</td>
<td>92</td>
</tr>
<tr>
<td>5.3.3 Cooperativity at the ABC-Enhancer</td>
<td>93</td>
</tr>
<tr>
<td>5.3.4 LFB₁ is able to Induce the ABC-Enhancer like LFB₃</td>
<td>94</td>
</tr>
<tr>
<td>5.3.5 Inducibility Observed on AAC and BBC</td>
<td>95</td>
</tr>
<tr>
<td>5.3.6 Does LFB₃ Expression for 15 Hours lead to Differentiation of F9 Cells?</td>
<td>95</td>
</tr>
<tr>
<td>5.3.7 Effect of CREB and ATF1 Overexpression in F9 Cells</td>
<td>96</td>
</tr>
<tr>
<td>5.3.8 Discussion</td>
<td>97</td>
</tr>
</tbody>
</table>

### 6. GENERAL DISCUSSION

<table>
<thead>
<tr>
<th>Sections</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Model for the Transcriptional Regulation of the ABC-Enhancer</td>
<td>99</td>
</tr>
<tr>
<td>6.2 Mechanism of Transcriptional Activation</td>
<td>99</td>
</tr>
<tr>
<td>6.2.1 Confirmation of CREB- and ATF1-Involvement in the ABC-Enhancer</td>
<td>99</td>
</tr>
<tr>
<td>6.2.2 Does LFB₃ Bind to CREB or ATF1?</td>
<td>100</td>
</tr>
<tr>
<td>6.2.3 Does ATF1 Interact with CBP?</td>
<td>101</td>
</tr>
<tr>
<td>6.2.4 To which TAFs Binds LFB₃?</td>
<td>101</td>
</tr>
<tr>
<td>6.2.5 Does the ABC-Enhancer exist in species other than pig?</td>
<td>101</td>
</tr>
<tr>
<td>6.3 The ABC-Site is a Prototype Enhancer for Coupling the Tissue-specificity and Hormonal Regulation of a Gene</td>
<td>102</td>
</tr>
</tbody>
</table>

### 7. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>103</td>
</tr>
</tbody>
</table>

### 8. REFERENCES

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.</td>
<td>105</td>
</tr>
</tbody>
</table>

### 9. CURRICULUM VITAE

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>121</td>
</tr>
<tr>
<td>9.1 Publications</td>
<td>122</td>
</tr>
</tbody>
</table>

### 10. ACKNOWLEDGMENTS

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.</td>
<td>123</td>
</tr>
</tbody>
</table>
1. SUMMARY

The urokinase-type plasminogen activator (uPA) is an extracellular protease which converts plasminogen into plasmin which is a protease with a broad substrate specificity. In this way, uPA participates in the control of many physiological and pathological functions that require the degradation of the extracellular matrix including wound healing, gametogenesis, metastasis formation and angiogenesis. The expression of uPA is controlled by many different extracellular signals as a consequence of its wide scope of activity.

In LLC-PK₁ cells, a cell line derived from pig kidney epithelia, the uPA gene expression is increased dramatically after stimulation of the cells with hormones leading to an increase in the intracellular concentration of cAMP. The site in the uPA promoter responsible for this induction is located 3.4 kb upstream of the transcription initiation site and is comprised of three adjoining protein-binding domains, A, B and C (the ABC-site). Domains A and B contain CRE (cAMP-response element)-like sequences but require the adjoining C domain to confer full cAMP responsiveness on a heterologous promoter. The C domain has no CRE and cannot mediate cAMP responsiveness when used in isolation. The protein binding to the C domain is the pig equivalent of the mouse LFB3. LFB3 is a tissue-specific transcription factor that is highly expressed in kidney cells.

In this study, the cis-elements and trans-acting factors required for the function of the ABC-site were characterized. In the first part of this thesis, the role of LFB3 in the control of the ABC-site is described. It was shown that mutations within the ABC-site abolished full cAMP-dependent gene induction. This led to the proposition that the three protein-binding domains act cooperatively in regulating uPA gene expression and that the presence of LFB3 is absolutely required for gene induction. This idea was tested in transient transfection assays using F9 cells which contain no endogenous LFB3. An ABC-linked luciferase reporter gene construct was transfected into F9 cells together with an expression vector of LFB3 and the cells were induced by cotransfection of an expression vector for the catalytic subunit of protein kinase A. Clear cooperation among the three domains and an absolute requirement for the presence of LFB3 was observed. To test for feedback regulation, LFB3 mRNA levels and DNA-binding activity to domain C in cell-extracts treated with cAMP were measured and found to be strongly reduced. This reduction was not due to a change in the template activity of the LFB3 gene because run-on transcription showed no significant reduction in LFB3 gene transcription. RNA synthesis inhibitor-chase experiments indicated that the down-regulation of LFB3 message was post-transcriptional and that ongoing RNA synthesis was required for the decrease. Similar effects on LFB3 levels were also observed for all other reagents including TPA, which induces uPA gene expression, indicating that LFB3 is also involved in feedback regulation and cross-talk regulation of the uPA gene through the ABC-site. This hypothesis is consistent with the observation of reduced cAMP-induction of the ABC-driven luciferase gene in cells that had been pretreated with TPA.

In the second part of this thesis the requirements for cooperation at the ABC-site, which factors bind to domains A and B and how gene activation is mediated by the ABC-site are discussed. In electromobility shift assays using LLC-PK₁ cell extracts, the
CRE-binding protein CREB and the CREB-related protein ATF1 were found to bind to domains A and B, respectively. These proteins are ubiquitously expressed and accordingly cooperation at the ABC-site was observed in all the cell lines transfected with expression vectors for LFB3 and the catalytic subunit of protein kinase A. The binding of CREB and ATF1 to domains A and B, but not the consensus CRE-sequence is increased after their phosphorylation by protein kinase A. The significance of the deviation of the CRE-like binding sites in domains A and B from the consensus CRE was investigated. When the CRE-like sequences were converted to consensus CRE sequences mutated domains AB, termed A*B*, could mediate cAMP inducibility even without the adjoining C domain. The conversion of the domain C sequence to the consensus LFB3 recognition sequence led to a strong increase in basal activity and cAMP-inducibility. These results suggest that the imperfect CRE sequence and the C sequence are required for the tight coupling of hormonal and tissue-specific regulation. The adenoviral oncoprotein 12S E1A has been reported to associate with the transcriptional adapter proteins p300 and CBP and to negatively affect CREB-dependent gene expression. While E1A repressed the cAMP induction of the consensus CRE-linked luciferase gene, it showed no effect on the ABC-linked luciferase gene. This result was confirmed by experiments with overexpressed CBP and similar results were obtained, implying that the ABC-construct uses a transcriptional activation different from the consensus CRE-mediated transcriptional activation. In an attempt to understand this difference the role of the transcriptional activation domain of LFB3 in the ABC-site was addressed. This domain was found to be required for transcription, implying that LFB3 can functionally substitute for CBP in the ABC-enhancer.

The results presented in this thesis show that the cooperation between transcription factors binding to one enhancer enables the integration of tissue-specific and hormonal gene regulation.
2. ZUSAMMENFASSUNG


Zum Studium der uPA-Genregulation eignet sich besonders die LLC-PK1-Zelllinie. Diese Zellen sezernieren uPA nach Behandlung mit Hormonen, die die Konzentration an zyklischem AMP (cAMP) erhöhen. Eine Erhöhung der cAMP-Konzentration in der Zelle führt zur Aktivierung des Protein Kinase A-abhängigen Signaltransduktionspfades.


In dieser Dissertation wird beschrieben, welche Faktoren für die volle Funktion des ABC-Enhancers nötig sind. Im ersten Teil wurde die Bedeutung von LFB3 in der Regulation des ABC-Enhancers untersucht. Es zeigte sich, dass Mutationen im ABC-Enhancer die cAMP-abhängige Geninduktion verhindern. Dieses Resultat führte zur Hypothese, dass die drei Domänen A, B und C in der Regulation des uPA-Gens kooperieren, und dass die Anwesenheit von LFB3 für die Geninduktion absolut nötig ist. Diese Hypothese wurde in einer Zelllinie getestet, die kein LFB3 enthält, nämlich der F9 Zelllinie. Durch Transfektion des Luciferase-Reportergens unter der Kontrolle des ABC-Enhancers, eines LFB3-Expressionsvektors und eines Vektors zur Expression der katalytischen Untereinheit der Protein Kinase A, anstelle der Induktion durch cAMP, konnte in F9 Zellen bewiesen werden, dass alle drei Domänen zur vollen Geninduktion nötig sind, und dass ohne LFB3 keine cAMP-abhängige Induktion erfolgt. Da eine Feedbackregulation durch LFB3 vermutet wurde, wurde die Menge an LFB3 mRNA und an DNA-Bindungsaktivität an die C-Domäne nach Behandlung der Zellen mit cAMP bestimmt und eine signifikante Reduktion festgestellt. Diese Reduktion an LFB3 mRNA Menge wurde nicht hervorgerufen durch eine Änderung der Transkriptionsrate des LFB3 Gens, sondern war ein posttranskriptioneller Effekt, der kontinuierliche mRNA-Synthese benötigte. Interessanterweise war diese Reduktion nicht nur auf cAMP beschränkt, sondern auch alle anderen Induktoren, die das uPA-Gen induzieren. Diese Daten implizieren, dass LFB3 für die Geninduktion nötig ist, jedoch langfristig eine erneute Geninduktion verhindert. Ausserdem können andere Faktoren,
die das uPA-Gen induzieren, die Menge an LFB3 reduzieren und dadurch die cAMP-
abhängige Induktion des uPA-Gens modifizieren.

Im zweiten Teil meiner Dissertation, wurden die Bedingungen für die Kooperation
am ABC-Enhancer charakterisiert, welche Proteine an die Domänen A und B binden,
und wie der ABC-Enhancer zur Geninduktion führt. Es stellte sich heraus, dass das
CRE-bindende Protein CREB und das verwandte Protein ATF1 an die Domänen A und
B binden können. Beide Proteine sind in allen Zellen vorhanden. Entsprechend konnten
alle getesteten Zelllinien den ABC-Enhancer induzieren, wenn sie mit einem
Expressionsvektor für LFB3 und Protein Kinase A transfiziert wurden. Ausserdem
wurde untersucht, warum in den Domänen A und B nur eine CRE-ähnliche und nicht
eine konsensus CRE Sequenz vorliegt. Durch Mutation der CRE-ähnlichen zur
konsensus CRE Sequenz war die Anwesenheit von LFB3 nicht mehr nötig. Genauso
bewirkte die Umwandlung der C-Domäne zur konsensus LFB3 Erkennungssequenz eine
Erhöhung der Basalaktivität. Diese Ergebnisse deuten darauf hin, dass die Abweichung
der Bindungssequenzen im ABC-Enhancer von konsensus Sequenzen für die Kopplung
von gewebspezifischer und hormoneller Regulation von Bedeutung ist. Weitere
Experimente weisen darauf hin, dass der Mechanismus für die transkriptionelle
Aktivierung verschieden ist, je nachdem ob ein Gen unter der Kontrolle einer konsensus
CRE-Sequenz oder des ABC-Enhancers ist.

Die Resultate in dieser Dissertation beschreiben ein System, in dem
Transkriptionsfaktoren an einem Enhancer zusammentreffen und dadurch
gewebspezifische und hormonelle Regulation an einem Enhancer integrieren.
3. GENERAL INTRODUCTION

3.1 Prologue

In a multicellular organism each cell contains the same genetic information, no matter if these cells are present in eyes, heart or liver. Since all cells contain the same genes and since the phenotype of a cell is determined by the expressed genes, it is a fundamental question of modern biology to understand, how the expression of genetic information is regulated. In every cell, some genes are constantly expressed. These genes are called housekeeping genes. Their gene products are required in all cell types. In contrast, a large number of genes are not expressed in all cells but only in cells of a certain organ. These gene products fulfill certain organ-specific functions such as secretion of a certain hormone or the regulation other cell-specific genes.

In a cell, a large number of genes are not expressed, i.e. these genes are inactive. Under certain conditions such an inactive gene can become active. This induction of the gene can be triggered by stimulation of the cell with a hormone. When a hormone interacts with its target cell, the signal is rapidly amplified by a whole cascade of proteins being modified and modifying others or leading to a change in the concentration of a certain substance in the cell, ultimately resulting in the induction or repression of a gene. How the cell reacts after receiving the extracellular signal is a cell-specific phenomenon, depending on the receptors expressed by the cell, the factors existing in the cell, the chromatin structure and the stage of the cell-cycle. Therefore the same signal can lead to different reactions in different cell types, although they have the same genome. Thus, cell-type specific gene expression is crucial for the development of cell-specific functions of a cell. Understandably much effort has been undertaken to elucidate mechanisms by which cell-specific gene expression is achieved.

In this dissertation, I describe a system where the urokinase-type plasminogen activator gene is specifically expressed in a kidney cell line after stimulation of the cell with the hormones calcitonin or vasopressin. The tissue-specific and hormonal regulation of the uPA gene is mediated in this system by an enhancer which has binding sites for tissue-restricted and hormonally regulated transcription factors. I have characterized the synergy between transcription factors which ensures tissue-specific uPA gene expression under hormonal control.
3.2 Gene Expression is Regulated on Different Levels

To control the expression of genes is one of the most important regulatory events in a cell. The presence, or absence, of a gene product can lead to subtle or drastic changes of the cell phenotype. Under certain conditions, the uncontrolled expression of cellular factors, e.g. oncogenes, can result in malignant transformation. Since gene regulation is so important, many control mechanisms exist that affect gene expression on different levels. In the control of gene expression, it is not only necessary to regulate the synthesis of the protein but also to regulate the breakdown of the protein and the mRNA.

mRNA is one of the intermediate products in the process of the synthesis of a protein since it transmits the genetic information from the nucleus to the cytoplasm and thereby connects these distinct compartments. mRNA is subject to control at many different levels as shown in Figure 1.

![Diagram of mRNA regulation](image)

Figure 1: The amount of mRNA in the cytoplasm is controlled at many different levels.

3.2.1 mRNA Stability is Regulated by Destabilizing Elements

The stability of mRNA plays a crucial role in the regulation of gene expression. The steady-state level of mRNA is the net result of its synthesis and decay. Therefore mRNA degradation contributes to the steady-state level of mRNA as much as its de novo synthesis. mRNA degradation is a regulated process, because the decay of mRNA is an important step in the control of gene expression. The stability of different mRNAs can
vary greatly within the same cell. For example the c-fos mRNA has a half-life of 20 minutes while the β-globin mRNA has a half-life of 24 hours (Peltz et al. 1992). mRNA stability can also be regulated by changes in cell growth rates and in the cell cycle according to the needs of the metabolism.

The general model for mRNA turnover states that mRNA is in principle stable but carries destabilizing elements which determine its degradation rate. Several types of destabilizing elements were identified. These elements are characterized by their ability to confer destabilization when inserted into a stable mRNA. The only sequences that have been found which confer stability to a normally unstable mRNA are the 5’cap and poly(A) tail of the mRNA and regions of mRNA which can form secondary structure and thereby stop the degrading enzyme (Vreken et al. 1992; Decker et al. 1993).

The destabilizing sequence elements are not localized in a particular region of the mRNA but are found throughout the whole message. The general structure of the mRNA is displayed in Figure 2.

![Figure 2: Structure of the mRNA](image)

- **5’UTR**
- **CDS**
- **3’UTR**

**Figure 2**: Structure of the mRNA. The spliced, eukaryotic mRNA has a cap at its 5’end followed by an untranslated region (UTR). The coding sequence (CDS) is initiated with an AUG and terminated with a termination signal (Term). At the 3’end a UTR is localized adjacent to a poly(A) tail of variable length.

### 3.2.1.1 The 5’Cap Structure

A 5’-terminal cap structure is a common feature of almost all eukaryotic mRNAs (Shatkin, 1976). The cap structure is important in the process of translation and in the regulation of mRNA stability. The unique 5’-5’ phosphodiester bond of the cap makes the mRNA intrinsically resistant to 5’-3’-exoribonucleases. Therefore, its removal may render mRNAs susceptible to the action of 5’-3’-exoribonucleases or alternatively, to an endoribonuclease whose target site was masked by the cap structure and its associated cap binding proteins (Shimotohno et al. 1977).

A decapping enzyme has been purified from yeast (Stevens, 1988) and activation of this enzyme by sequence elements of the mRNA could create unstable mRNA by making it susceptible to 5’-3’-exoribonucleases. The existence of a 5’-3’-exoribonuclease has been confirmed by the purification of the XRN1 protein from yeast (Larimer et al. 1990).
3.2.1.2 The Destabilizing Elements Located in the 5' UTR

The 5' untranslated region (UTR) has not yet been definitively shown to contain destabilizing elements. It appears to affect mRNA stability by changing the rate of translation of the mRNA. Often hairpin structures in the 5' UTR cause the polysomes to stop or to slow when translating the mRNA. Most mRNAs need to be translated to be degraded. Therefore, inhibition of translation by hairpin structures at the 5' UTR can cause stabilization of the message.

Exposure of cells to cycloheximide, an inhibitor of polypeptide chain elongation, leads to the accumulation of normally unstable mRNAs. This is apparently due to message stabilization. One explanation for this effect is that a protein component of the decay machinery is unstable and disappears rapidly when protein synthesis is blocked. Some experimental evidence for the occurrence of such an unstable cytoplasmic factor has been presented by Brewer et al. (1989).

3.2.1.3 Destabilizing Elements in the Coding Sequence

As mentioned earlier, sequence elements which destabilize mRNA are found throughout the whole mRNA. Accordingly, several destabilizing elements are located in the coding sequence. One example is the tubulin mRNA. The tubulin synthesis in cells is tightly controlled by measuring the unassembled tubulin subunit concentration (Cleveland et al. 1981). Tubulin synthesis is regulated posttranscriptionally. An elevation of the amount of β-tubulin subunits leads to a decreased stability of β-tubulin mRNA, thereby reducing the amount of β-tubulin subunits in the cell. Therefore tubulin synthesis is an autoregulated process. Protein synthesis inhibition by pactamycin and puromycin which cause premature peptide chain release prevents this autoregulated degradation of β-tubulin mRNAs. In contrast, a low concentration of cycloheximide, which slows ribosome translocation but leaves polysomes intact, actually enhanced autoregulated instability. Thus, only β-tubulin mRNAs attached to polysomes are substrates for autoregulated degradation. Using a series of constructs Yen and coworkers showed that the first thirteen translated codons are responsible for changes in the stability of β-tubulin mRNA with the first four amino acids (MREI) being essential for autoregulation (Yen et al. 1988).

The c-fos mRNA contains destabilizing elements in the 3' UTR and the coding sequence. It is very unstable and decays rapidly in the cytoplasm with a half-life of less than 30 minutes. This pronounced instability distinguishes it from most mammalian mRNAs, whose half-lives range from hours to days. Two distinct cellular pathways for rapid c-fos mRNA degradation exist (Shyu et al. 1989). Each of these pathways
recognizes a different, functionally independent instability determinant. One of these is located within the 3′ UTR whereas several other destabilizing elements are found in the coding region (Wellington et al. 1993). Translation of the c-fos mRNA is required for inducing destabilization and the decay does not depend on the protein but on mRNA itself.

3.2.1.4 The Destabilizing Elements in the 3′ UTR

The best studied example of regulated mRNA decay is a class of short-lived mRNAs. Their common feature is the presence of the repeated sequence (AUUUA)$_n$ also called the AU-rich element (ARE), in the 3′ UTR. It was demonstrated by Shaw et al. (1986) that insertion of the ARE from the 3′ UTR of GM-CSF mRNA into that of β-globin specifically reduced the half-life of globin mRNA from 17 hours to less than 30 minutes. Thus, the presence of an ARE in a mRNA can cause the mRNA to be degraded very rapidly. The ARE functions as a destabilizing element whose activity is regulated by extracellular stimuli.

The 3′ UTR of uPA mRNA contains at least three regulatory sites that are responsible for its short half-life of 70 minutes. One of the three elements is an ARE which mediates uPA mRNA stabilization induced by protein kinase C downregulation (Nanbu et al. 1994). A cytoplasmic factor of 40 kDa (p40) binds to this ARE. The expression level of p40 is increased in PKC-downregulated LLC-PK$_1$ cells compared to nontreated cells. The p40 protein is most likely identical to hnRNP C. In a human breast cancer cell line MDA231, uPA mRNA has a much longer half-life than in LLC-PK$_1$ cells. p40 is present in higher concentrations in MDA231 cells than in LLC-PK$_1$ cells indicating that p40 is involved in cell-specific stabilization of the uPA mRNA.

The ARE in the 3′ UTR of c-fos mRNA functions as a destabilizing element whose activity is not affected by growth factors or cytokine stimulation. The differential regulation of the stability of mRNAs containing the c-fos and GM-CSF AREs shows that, while these two elements are both AU-rich, there must be some important sequence difference that accounts for their distinct behavior. One difference between these two classes of ARE is that the ARE of the GM-CSF like mRNAs contains three or more contiguous (AUUUA)$_n$ motifs, while those in the protooncogene mRNAs are generally scattered throughout the element. These two types of ARE bearing 3′ UTRs can be independently regulated in the same cell type. This leads to the idea that different trans-acting factors can differentially recognize the two classes of ARE and that the two classes of ARE have distinct functions.
Several distinct protein factors have been characterized that bind to ARE. They have been found in the cytoplasm and in the nucleus. The presence of ARE-binding proteins in the nucleus suggests that the ARE may have a function in the nucleus too or in transport from the nucleus to the cytoplasm.

Iron uptake in cells is mediated by transferrin and the transferrin-receptor (TfR). The TfR expression is regulated according to iron availability in a manner suggestive of a negative feedback regulation. Thus, fewer receptors are expressed when iron is abundant and more receptors are expressed when iron is scarce. The iron-dependent alterations in TfR biosynthesis have been found to be mediated by alterations in the level of TfR mRNA (Mattia et al. 1984). The steady-state level of TfR mRNA has been found to increase after treatment of cells with an iron chelator and to decrease after exogenous iron was supplied (Rao et al. 1986). The regulation of TfR mRNA levels is partly due to a change in gene transcription rate. However, the predominant locus of iron regulation of TfR expression lies not in the promoter, but in sequences located in the 3'UTR of the TfR mRNA (Casey et al. 1988). The information for the iron-regulated changes in TfR mRNA stability has been localized to a portion of the 3'UTR forming five stem-loop structures strikingly similar to the ferritin IREs. Further studies have demonstrated that intact IREs are required to regulate the stability of the TfR mRNA (Casey et al. 1989).

To understand the mechanism by which iron levels are sensed, cellular factors interacting with this RNA structure have been identified and characterized. One of them, the IRE-binding protein (IRE-BP), has been found to change its affinity for IRE according to cellular iron levels. These changes in affinity occur through dissociation and reassociation of an iron-sulfur cluster within the IRE-BP. If iron is scarce, the IRE-BP is bound to the IRE and thereby represses degradation. Interestingly, the IRE-BP also possesses aconitase activity, suggesting an unusual but possibly important linkage between general cellular metabolism and RNA metabolism.

Deletions within the regulatory region support a model of TfR mRNA regulation that envisions two distinct functions within the regulatory region: IREs and an iron-independent instability determinant. The instability element mediates the rapid destruction of the transcript, and the function of this rapid turnover determinant is unimpeded as long as the IREs are not occupied by an IRE-BP. However, occupation of the IREs by the high affinity IRE-BP, which are dispersed throughout the regulatory region, prevents association of destabilizing factors to the instability determinant. Whether these destabilizing factors are themselves ribonucleases or whether they form a complex with ribonucleases to destroy the mRNA at a distinct site is as yet unknown.
The regulation of TfR mRNA decay provides important mechanistic insights: first, it shows how the decay process can be regulated by proteins that can directly respond to changes in the cellular environment; second, mRNA stabilization may in fact result from an inhibition of an activity recognizing the destabilizing element; and third, the destabilizing element can be distant from the binding site of the stabilizing proteins.

3.2.1.5 The Poly(A) Tail

mRNAs are deadenylated before degradation, meaning that the poly(A) tail is usually removed before mRNAs are degraded (Decker et al. 1993). The poly(A) tail lengths of specific mRNAs were measured and poly(A) tails were found to be absent below a certain minimal length, which is usually estimated to be about 10-30 adenylates (Muhlrad et al. 1992). Thus, the poly(A) tail shortening process seems not to lead to completely deadenylated mRNAs in most cases; rather, mRNAs with short poly(A) tails (also called oligo(A) tails) accumulate as the major end products. The poly(A) shortening to the stable, minimal length and complete removal may therefore represent distinct processes (Lowell et al. 1992). The poly(A) tail is not only important in mRNA turnover but also in regulating translational efficiency of the mRNA.

3.2.1.6 Trans-Acting Factors Involved in mRNA Decay

Trans-acting factors that are likely to play a role in mRNA turnover include specific endo- and exoribonucleases as well as factors that bind to specific mRNA sequence elements to promote or inhibit the initiation of decay, to target the mRNA to a specific site or to regulate mRNA translational efficiency. Trans-acting factors have been identified primarily by biochemical approaches.

Many proteins have been characterized which interact with the poly(A) tail. The poly(A) binding protein (PABP), a ~70 kDa protein, has been found to be common to most, if not all, eukaryotes. In yeast, it has been possible to demonstrate that the PABP gene is essential for viability. Binding of PABP to poly(A) is very stable even in high salt concentrations. Digestion of poly(A)-PABP complexes with non-specific nucleases results in fragments with PABP bound to a length of 12 nucleotides (Sachs et al. 1987). Therefore, oligo(A) tails may be unable to bind efficiently to PABP.

From yeast, Sachs and Deardorff have purified and characterized the poly(A) nuclease (PAN), an exoribonuclease (Sachs et al. 1992). The PAN gene encodes a 161 kDa polypeptide containing several repeated sequences. The gene is essential and PAN- mutants are defective in translational initiation. PAN has been shown to require the presence of PABP in order to degrade poly(A) exonucleolytically. The PAN-PABP
complex normally shortens to about 15 nucleotides. It should be emphasized that the need of a nuclease for a RNA binding protein to degrade RNA with full efficiency is unique among identified eukaryotic RNases. Interactions could be regulated by other proteins or many mRNAs. PAN reveals some interesting properties which may be more general for RNA degradation.

The mRNA is not protected from degradation by RNA-binding proteins. In contrast, the presence of PABP forming a ribonucleoprotein complex with the mRNA is the substrate for PAN. Therefore the search for RNases that specifically recognize sequences within mRNA that have been found to have a destabilizing effect may be unsuccessful unless the ribonucleoprotein substrate is identified. Furthermore, one can conclude that destabilizing sequences can activate or inhibit the activity of nucleases over long distances.

Thus, destabilizing sequences located throughout an mRNA molecule can affect deadenylation and decay pathways. The requirement for PAN in translation and RNA decay leads to the suggestion that some of the factors involved in mRNA decay may also be complexed with translation initiation factors.

In contrast to the PABP, several ARE binding proteins have only been identified in vitro. One of these factors, Auf, which has been purified from the cytosol of k562 erythroleukemia cells, appears to contain two distinct polypeptides 37 and 40 kDa in size. Auf binds to the AREs of both c-myc and GM-CSF mRNA (Brewer, 1991). Moreover, Auf stimulates the decay of c-myc mRNA in an in vitro cell-free system. The observation that Auf can accelerate the process of mRNA decay in vitro lends support to the idea that it may be involved in controlling mRNA degradation in vivo. The existence of multiple ARE binding factors, sometimes coexisting within a single cell, suggests an intriguing degree of complexity for ARE function. Additional studies will be required to establish the importance of these various factors for the decay or stabilization of ERG mRNAs in vivo.

Other mRNA-binding proteins likely to have a role in mRNA decay include the HTLV-1 p27rex and a 66 kDa chicken protein (Kanamori et al. 1990). They appear to protect their respective binding sites from endonucleolytic cleavage, an activity that may be regulated by the translation state of the mRNA.

3.2.1.7 Possible Mechanisms for mRNA Decay

The first step in mRNA degradation seems to be the shortening of the poly(A) tail to a length of about 10 nucleotides. Alterations in the rates of poly(A) tail shortening can regulate mRNA decay since poly(A) removal appears to be rate limiting in the decay
process. Once deadenylation has proceeded to a tail length of about 10 nucleotides, further deadenylation by 3'-5' exonucleases is likely but possibly not compulsory for the next step in the decay (Decker et al. 1993). The mRNA is degraded after being shortened to the oligo(A) form. Deadenylation to an oligo(A) tail could stimulate degradation by affecting the efficiency of translational initiation, the binding of other proteins or the RNA conformation. Because individual mRNAs show differences in the rate of this final step, it will be important to identify the rate-determining factors in the decay of the oligo(A) species (Decker et al. 1993). Two mechanisms of the further decay have been proposed: internal cleavage (Shatkin, 1976); and removal of the 5' cap structure by cleavage of the pyrophosphate bond between the cap and the mRNA (Shimotohno et al. 1977; Stevens, 1988). In both cases, these events would be followed by 5'-3' exonucleolytic digestion (Beelman et al. 1995).

There are two general explanations why deadenylation would stimulate cleavage events elsewhere on the mRNA. Poly(A) tail shortening could decrease the rate of translational initiation of this mRNA (Sachs et al. 1989). This could then leave the mRNA susceptible to cleavage. Alternatively, deadenylation may have a more direct effect by disrupting interactions between the 3' end of mRNA and other sites on the RNA molecule that are dependent on the poly(A) tail (Galili et al. 1988). Loss of such interactions could lead to alterations in mRNA structure, thereby exposing sites susceptible to cleavage within or at the 5' end of the mRNA.

A mechanism of action of destabilizing sequences within mRNA can be hypothesized from existing data. These sequences require translation for activity since their recognition is mediated by factors brought to the mRNA by the translational machinery. Once recognized, these elements induce decay by activating poly(A) tail removal. Following the terminal deadenylation step, identical or other sequence elements within mRNA are recognized by the exo- or endonucleases that eventually lead to the destruction of the mRNA.

A more complete understanding of mRNA decay will require more detailed descriptions of the sequences that determine instability within mRNAs and an understanding of why there is a requirement for translation in RNA degradation.
3.3 Regulation of Eukaryotic Gene Transcription by cAMP

Many hormones, neurotransmitters and growth factors are known to bind to cell surface receptors which cause an increase in intracellular cAMP-concentration. Cyclic AMP participates in the control of many important metabolic processes ranging from the breakdown of fat in adipocytes and of glycogen in the liver to the synthesis of hormones, such as somatostatin in the hypothalamus.

An extracellular signal in the form of a hormone or neurotransmitter meets a specific binding site on the cell-surface. The binding site of the extracellular signal is a receptor with seven membrane-spanning regions. Binding of the hormone to the receptor leads to activation of the trimeric G-proteins by exchange of GDP-moiety on the α-subunit of the G-protein with a GTP. The GTP-bound α-subunit meets the membrane-linked adenyllylcyclase, which converts ATP to cAMP. Cyclic AMP, in turn, activates protein kinase A (PKA), which is located by the regulatory (R) subunit of PKA to specific sites in the cytoplasm, e.g. microtubules (reviewed in Mochly-Rosen, 1995). The holoenzyme form of PKA exists as an inactive tetrameric complex, with two regulatory and two catalytic subunits, R$_2$C$_2$. There are two major isoforms of mammalian PKA (type I and II) which have essentially the same C subunits, but have distinct R subunits. The two PKA isoforms have different specificities for cAMP analogues. cAMP binds to two sites of the R subunit of PKA, thereby releasing the C subunit of PKA, which translocates from the cytoplasm to the nucleus. In the nucleus, the PKA phosphorylates substrates with the motif X-Arg-Arg-X-Ser-X at the serine.

3.3.1 The Nuclear Target of PKA

Several nuclear factors are involved in regulation of gene expression by cAMP. Proteins involved in cAMP-dependent gene regulation were identified by their ability to bind to a sequence element found in promoters of genes responding to an increase in intracellular cAMP concentration. The sequence element which mediates cAMP-responsiveness is termed the cAMP-response element (CRE) and is described in section 3.3.3.

3.3.1.1 The CREB/ATF-Family

The factors which bind to the CRE all belong to the CREB/ATF family which has a large number of members. The prototype of this family is CREB, the cAMP-response element binding protein (Montminy et al. 1995). Most of the CREB/ATF-family members are shown in Table 3-1. Several CREB/ATF-family members bind to the CRE strongly, others bind weakly and bind only to triple CREs or preferentially bind to the
TPA-response element (TRE). Only the activating transcription factor-1 (ATF1), the CRE-modulators (CREMs) and CREB-341 and its splice variants are phosphorylated by PKA and are able to bind with a high affinity to CREs. CREB, ATF1 and CREM are expressed in a variety of different cell lines (Montminy et al. 1987; Hai et al. 1989; Rehfuss et al. 1991; Laoide et al. 1993). The other proteins of the CREB/ATF-family are not reported to be involved in cAMP-signaling, but in other signal transduction pathways such as those involving MAP-kinase [e.g. ATF2 (Abdel-Hafiz et al. 1992) and ATF3 (Tan et al. 1994)], TNFα [ATF2 (Tsai et al. 1996)] or Jun-kinase [e.g. ATF2 (Chen et al. 1996) and ATF3 (Gupta et al. 1995)]. Thus, CREB, ATF1 and CREM are functionally distinct from the rest of the CREB/ATF-family, since CREB, ATF1 and CREM bind to CREs with high affinity and since they are the substrates of PKA. Therefore, CREB, ATF1 and CREM form a subfamily of the CREB/ATF-family and are involved in cAMP-signaling.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>ref</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB-341</td>
<td>PC12</td>
<td>1</td>
<td>also named CREB1</td>
</tr>
<tr>
<td>CREB-327</td>
<td>placenta</td>
<td>2</td>
<td>isoform of CREB-341, lacks α-domain</td>
</tr>
<tr>
<td>CRE-BP1</td>
<td>brain</td>
<td>3</td>
<td>also named CREB2, identical to ATF2</td>
</tr>
<tr>
<td>CRE-BPa</td>
<td>KG-1 cells</td>
<td>4</td>
<td>splice variant of CRE-BP1</td>
</tr>
<tr>
<td>mXBP</td>
<td>B cells</td>
<td>5</td>
<td>identical to ATF2</td>
</tr>
<tr>
<td>HB16</td>
<td>B cells</td>
<td>6</td>
<td>identical to ATF2</td>
</tr>
<tr>
<td>ATF1</td>
<td>HeLa</td>
<td>7</td>
<td>stimulates transcription after cAMP and Ca(^{2+})-influx, also named CREB B</td>
</tr>
<tr>
<td>ATF2</td>
<td>HeLa</td>
<td>7</td>
<td>stimulates transcription after viral induction</td>
</tr>
<tr>
<td>ATF3</td>
<td>HeLa</td>
<td>7,8</td>
<td>stress-induced, homodimers repress, ATF3/Jun heterodimers stimulate transcription</td>
</tr>
<tr>
<td>ATF4,5,6</td>
<td>HeLa</td>
<td>7</td>
<td>30-40% homologous to CREB-327</td>
</tr>
<tr>
<td>ATF5,6</td>
<td>HeLa</td>
<td>7</td>
<td>ATF5, 6 bind only to 3xCRE weakly</td>
</tr>
<tr>
<td>ATF7</td>
<td>HeLa</td>
<td>7</td>
<td>binds to CRE, but not to TRE</td>
</tr>
<tr>
<td>ATF8</td>
<td>HeLa</td>
<td>7</td>
<td>Jun-like, binds to TRE, binds weakly to 3xCRE</td>
</tr>
<tr>
<td>ATF-a</td>
<td>HeLa</td>
<td>9</td>
<td>related to ATF2</td>
</tr>
<tr>
<td>ATF-aΔ</td>
<td>HeLa</td>
<td>9</td>
<td>splice variant of ATF-a</td>
</tr>
<tr>
<td>ATF-47</td>
<td>HeLa</td>
<td>10</td>
<td>identical to CREB-341</td>
</tr>
<tr>
<td>ATF-43</td>
<td>HeLa</td>
<td>10</td>
<td>related to ATF-47</td>
</tr>
<tr>
<td>B-ATF</td>
<td>B cells</td>
<td>11</td>
<td>related to ATF3, binds to TRE</td>
</tr>
<tr>
<td>CREM</td>
<td>pituitary gland</td>
<td>12</td>
<td>related to CREB-341, positive and negative regulator of cAMP-induced transcription</td>
</tr>
</tbody>
</table>

Table 3-1: The CREB/ATF-family. In bold are the proteins which are known to be phosphorylated by PKA. TRE = TPA response element. The references are: (1) (Montminy et al. 1987), (2) (Hoeffler et al. 1988), (3) (Maekawa et al. 1989), (4) (Nomura et al. 1993), (5) (Ivashkiv et al. 1990), (6) (Kara et al. 1990), (7) (Hai et al. 1989), (8) (Liang et al. 1996), (9) (Gaire et al. 1990), (10) (Hurst et al. 1990), (11) (Dorsey et al. 1995), (12) (Foulkes et al. 1991).
3.3.1.2 The Basic Leucine Zipper

All of the proteins in Table 3-1 belong to the basic leucine zipper (bZIP)-family. Other members of this family are the Jun- and Fos-related proteins. The bZIP is a structural motif which mediates dimer formation and DNA-binding. This motif is a basic region located towards the N-terminus of a leucine-rich area. This leucine-rich domain forms a α-helix. With the basic part of the bZIP, DNA-binding occurs and with the immediately adjoining leucine zipper the proteins form homo- or heterodimers. The leucines are always exposed in one direction allowing the interaction with the leucines of a second bZIP-protein (Busch et al. 1990). Not all combinations of heterodimers of bZIP-proteins are allowed, as summarized in Table 3-2. The subgroup of the CREB, CREM, ATF1-proteins can heterodimerize among each other. Therefore these proteins, which are involved in cAMP-signaling, can heterodimerize with each other but not with other members of the CREB/ATF-family which are not involved in cAMP-signaling. Similarly, proteins of the CREB/ATF-family which do not belong to the CREB, CREM, ATF1-subfamily can form heterodimers with each other and with Jun-related proteins but not with the CREB, CREM, ATF1-subfamily. Therefore, heterodimerization by the leucine zipper motif is very selective. The structural rules that govern the specificity of the dimerization are described elsewhere (Vinson et al. 1993).

<table>
<thead>
<tr>
<th></th>
<th>CREB</th>
<th>CREM</th>
<th>ATF1</th>
<th>ATF2</th>
<th>ATF3</th>
<th>c-Jun</th>
<th>c-Fos</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB</td>
<td>+1</td>
<td>+2</td>
<td>+3</td>
<td>-9</td>
<td>n.d.</td>
<td>-9</td>
<td>-9</td>
</tr>
<tr>
<td>ATF1</td>
<td>+3</td>
<td>n.d.</td>
<td>+5</td>
<td>-5</td>
<td>-5</td>
<td>5,10</td>
<td>6,7</td>
</tr>
<tr>
<td>ATF2</td>
<td>-9</td>
<td>n.d.</td>
<td>-5</td>
<td>-5</td>
<td>+5</td>
<td>6,8</td>
<td>6</td>
</tr>
<tr>
<td>ATF3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>5,10</td>
<td>+5</td>
<td>+5</td>
<td>6,11</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3-2: Heterodimer formation among some bZIP-family members. The two classes of leucine zippers allow the formation of only certain heterodimers. The references are: (1) (Liu et al. 1993), (2) (Laoide et al. 1993), (3) (Rehfuss et al. 1991), (4) (Foulkes et al. 1991), (5) (Hai et al. 1989), (6) (Hai et al. 1991), (7) (Masson et al. 1993b), (9) (Benbrook et al. 1990), (10) (Chen et al. 1996), 11 (Tan et al. 1994). n.d. = not determined.

3.3.1.3 Structure and Function of CREB

The structure of CREB is shown in Figure 3. The bZIP is located at the C-terminus. Two glutamine-rich domains are located at the N-terminus of the protein and N-terminal of the bZIP-domain. The serine is located in the middle of the protein, at position 133, and this is the substrate of the protein kinase A (Gonzalez et al. 1989). This serine is located in the kinase-inducible domain (KID, also called P-box). Apart from the PKA-phosphorylation site, the KID-domain contains two sequences important for CREB transcriptional activation, termed PDE-1 and -2 (Lee et al. 1990). PDE-1 is located towards the N-terminus, PDE-2 towards the C-terminus of the Ser133 (see Figure 4).
Constructs with deletions in PDE-1 or -2 are equally well phosphorylated by PKA at Ser133. The PDE-1 region is an area of phosphorylation by casein kinase II (Lee et al. 1990). The PDE-1 is required for the transactivation function induced upon phosphorylation of Ser133; deletion of PDE-1 completely abolishes cAMP-responsiveness. Therefore, PDE-1 is involved in cAMP-dependent regulation of CREB to the same extent as the PKA-phosphorylation site. An α-peptide region of 14-amino acids is located between KID and Q1 in CREB-341. In CREB-327, the α-peptide region is missing. Deletion of the α-peptide region diminishes transactivation potential (Yamamoto et al. 1990).

To determine the importance of the phosphorylation of serine 133, wild-type and mutant forms of CREB proteins containing substitutions at this residue were compared. The mutation of Ser133 to Ala133 completely abolished PKA-responsiveness. Similarly, substitution mutations to acidic amino acids, Ser133 to Asp133 or to Glu133, did not produce active proteins, suggesting that the negative charge provided by phosphorylation is not sufficient to stimulate transcription. Phosphorylation at Ser133 does not induce nuclear transport of CREB protein (Gonzalez et al. 1989). The phosphorylation at Ser133 most probably evokes a conformational change of CREB, leading to exposure of certain parts of the protein which could then activate transcription (Montminy et al. 1995).

Some kinases other than PKA can phosphorylate CREB, most notably Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaM kinases) I and II, casein kinase II (CKII) and glycogen synthase kinase-3 (GSK-3). CaM kinases phosphorylate CREB \textit{in vitro} at Ser133. The ability of both CaM kinases and PKA to phosphorylate CREB on the same
site provides the basis for Ca\(^{2+}\) and cAMP-dependent CREB activation (Dash et al. 1991; Sheng et al. 1991). However, it is unclear whether Ser133 phosphorylation alone is sufficient for gene induction. When Jurkat T-cells were stimulated at their T-cell receptor, an event which resulted in an increase in intracellular Ca\(^{2+}\)-concentration, a target gene was not induced unless cAMP was provided. Thus, phosphorylation at Ser133 mediated by an increase in intracellular Ca\(^{2+}\)-concentration may not be sufficient for gene induction (Brindle et al. 1995).

Phosphorylation by GSK-3 requires the prior phosphorylation of CREB by PKA at Ser133, creating the consensus site of the GSK-3 enzyme: \(SXXXS(P)\). Ser129 can then be phosphorylated, resulting in an increase in cAMP-dependent transcription, as assayed in F9 cells cotransfected with GSK-3\(\beta\) kinase (Fiol et al. 1994). Phosphorylation of CREB by GSK-3 probably provides means to fine tune cAMP-dependent signal transduction in the context of other signal-transduction pathways activated at the same time in the cell. Regulation of GSK-3 activity by phosphorylation occurs by different signal transduction pathways. Growth factors and hormones, most notably insulin, are involved in the control of GSK-3 activity through distinct signaling pathways including PI 3-kinase, PKB and p90 S6 kinase (reviewed in Welsh et al. 1996). Casein kinase II phosphorylates CREB at several residues including Ser111, Ser114, Ser117 and Ser156. Most of these serines are located in the PDE-1. As mentioned before, deletion of the PDE-1 abolishes cAMP-responsiveness but not phosphorylation by PKA, suggesting that the serines in PDE-1 are involved in the transactivation of CREB by cAMP. However, the function of the serines in PDE-1 is controversial (Lee et al. 1990; Montminy et al. 1995).

### 3.3.1.4 Structure and Function of ATF1 and CREM

The general structure of ATF1 and of the activator CREMt is shown in Figure 3. The KID-domain of ATF1 and CREM are similar to CREB: the three proteins, ATF1, CREB and CREM are 55% homologous in the N-terminal part of the KID, and 93% homologous in the C-terminal region (Masson et al. 1993b). The difference in the N-terminal part of the KID-domain, i.e. in the PDE-1, between ATF1 and CREB is intriguing, since this difference could have implications in different transactivation properties of the two proteins.

The homology between ATF1 and CREB at the bZIP is 91%, i.e. in the leucine zipper only two amino acids are not identical (Rehfuss et al. 1991). This supports the finding that CREB and ATF1 can heterodimerize. ATF1 does not contain the \(\alpha\)-peptide region that distinguishes CREB-341 from CREB-327.
In the KID-domain of ATF1 and CREM, the sequences of which are shown in Figure 4, there is a serine in a consensus PKA-motif similar to Ser133 of CREB. This residue is located at Ser63 in ATF1 (Hai et al. 1989) and at Ser117 in CREM (de Groot et al. 1993). In contrast to CREB and CREM, there is no GSK-3 site present in ATF1. ATF1 contains CKII consensus sites like CREB and CREM (Masson et al. 1993b). Like CREB, ATF1 can be phosphorylated when the intracellular Ca\(^{2+}\)-concentration is increased by CaM kinases I and II (Liu et al. 1993). Most remarkably, ATF1 undergoes a strong change in conformation upon phosphorylation, since treatment with phosphatases drastically changes the mobility of ATF1 on SDS-gels. In contrast, the mobility of CREB is not affected under the same conditions (Masson et al. 1993b). The N-terminal region (NTR) is important for the phosphorylation-induced change in mobility, although the phosphorylation sites are outside the NTR. Further analysis has shown that the Ser36 is most responsible for this conformational change, and this serine is absent in CREB and CREM, where it is replaced by Ala106 or Ala92, respectively. Additionally, Ser38 and 41 of ATF1 account for the induced conformational change. Ser36 can be phosphorylated by CKII. However, the physiological significance of these data are not clear. But it can be assumed that, despite the same binding specificities of CREB, CREM and ATF1, the regulation of these three proteins by kinases and phosphatases is different. Therefore, the interaction of the protein with DNA is altered depending on the phosphorylation state. Thus, the binding of certain proteins to CRE is likely to be favored depending on the signal transduction pathways activated at the time in a cell. In fact, recent in vitro experiments support the idea that ATF1, but not CREB is activated by CaMK II and that ATF1 phosphorylated by CaMK II can induce CRE gene expression. At the same time, CREB seems to be rather involved in PKA dependent CRE gene expression (Shimomura et al. 1996). These differences are ascribed to different serines being phosphorylated by CaMK II and PKA. A similar case
for c-Jun has been described, where the binding of c-Jun is altered depending on the residues phosphorylated (Boyle et al. 1991; Lin et al. 1992).

3.3.1.5 Isoforms of CREB, CREMs and ATFI

Several isoforms of CREB are generated by alternative splicing. Most of them vary in tissue-distribution and are differentially expressed during development (Yamamoto et al. 1990; Ruppert et al. 1992; Blendy et al. 1996). These isoforms have similar DNA-binding activities and transactivation potentials. The expression of certain CREB-isoforms is suddenly increased during spermatogenesis (Ruppert et al. 1992). A similar switch in splicing occurs with CREM during spermatogenesis (Foulkes et al. 1992; Delmas et al. 1994; Monaco et al. 1995). Several CREM isoforms are described and, except CREMα, all are repressors of CRE-dependent gene transcription (Laoide et al. 1993). CREB and CREM may have arisen from a common gene by gene duplication. This is supported by the finding of a predecessor of the mammalian CREM and CREB genes in Drosophila. The Drosophila gene product acts as a cAMP-responsive transcriptional activator and a splice product of the same gene is a specific antagonist of cAMP-inducible transcription (Yin et al. 1995b). For ATF1 no isoforms have yet been described.

3.3.1.6 Interaction between CREB and the Basal Transcription Factors

To understand how CREB activates transcription, the isolated KID and Q2 domains were tested in transient transfection assays. The deletion of Q2 in CREB completely abolishes CREB protein activity in response to cAMP. Q2 without KID fused to a DNA-binding domain, inducing strong promoter activity which was not controlled by cAMP. In contrast, KID fused to a DNA-binding domain had little basal activity but was inducible by cAMP. Therefore, KID and Q2 synergize to mediate cAMP-dependent gene induction, where the Q2 domain functions as a constitutive activator and the KID provides cAMP-responsiveness (Brindle et al. 1993). Therefore, an intramolecular coupling of two different functions provides cAMP-dependent gene activation. The fact that Q2 acts as a transcriptional activation domain is supported by its resemblance to the Spl activation domain.

The Q2-domain specifically interacts with Drosophila dTAFII110 protein, but not with other proteins of the TFIID-complexes, such as TBP, TAF40 and TAF80 (Ferreri et al. 1994). The amino acids of CREB that interact with dTAFII110 are most likely residues 204-208. This motif is absent from the CREMα and β-repressors. A similar interaction between CREB and TFIID has been described elsewhere, but an additional
interaction between CREB and TFIIB has been detected (Xing et al. 1995). Neither report detected a direct interaction between CREB and TBP (Ferreri et al. 1994; Xing et al. 1995).

### 3.3.2 Role of CBP in the cAMP-Signal Transduction Pathway

In an attempt to identify proteins that interact with phosphorylated CREB, a human λgt11 library was screened with phosphorylated CREB. The protein identified was termed CREB-binding protein (CBP) (Chrivia et al. 1993). CBP is a very large protein with an apparent molecular weight of 265 KDa and is mainly localized in the nucleus. CBP binds CREB with a domain situated between amino acids 462-661. A PKA-phosphorylation site of CBP is located at Ser1772. Whether this PKA-phosphorylation site is relevant for transactivation remains unclear, but recent results suggest that CBP-phosphorylation by PKA enhances transcriptional activation and antagonizes a putative phosphorylation by MAP-kinase (Janknecht et al. 1996). CBP has all the characteristics of a coactivator for CREB, since it does not bind to DNA itself and can contact the basal transcription factors through a region in the C-terminus (Kwok et al. 1994). Consistent with its role as coactivator, CBP specifically binds only to phosphorylated CREB and can augment the activity of phosphorylated CREB to activate cAMP-dependent gene transcription, but it does not regulate the nuclear targeting properties of CREB. In NIH 3T3 cells, an injected CRE-lacZ reporter gene was induced by treatment of the cells with 8-Br-cAMP and IBMX. Coinjection of antibodies against CBP reduced markedly the CRE-lacZ reporter activity (Arias et al. 1994). Thus, CBP is essential in the regulation of genes by cAMP.

Due to the high homology between CREB and ATF1 it is tempting to speculate that CBP interacts equally well with ATF1 as with CREB. In fact, CBP can bind to CaMK II phosphorylated ATF1 (Shimomura et al. 1996). However, ATF1 activity is not significantly stimulated by CBP in F9 cells. Only if the C-terminus of CBP is deleted, the truncated CBP is capable of acting as a positive regulator of transcription supporting the idea that the C-terminus of CBP has a regulatory role (Bisotto et al. 1996). This result is confirmed by two-hybrid assays where the CBP-homologue, p300, was shown to bind to CREB but not to ATF1 (Lee et al. 1996). Thus, CBP regulates transcription by ATF1 and CREB in a different way.

CBP contacts the basal transcription machinery. The C-terminal region of CBP is glutamine-rich and several factors are shown to bind to this region. With this glutamine-rich domain, CBP contacts the basal transcription factor TFIIB through a domain conserved in the yeast coactivator ADA-1 (Kwok et al. 1994). The importance of this
site to contact TFIIB is controversial, since recent data indicate that the C-terminus of CBP is dispensable for function (Bisotto et al. 1996). In contrast, an additional transcriptional activation domain located at the N-terminus was described (Bisotto et al. 1996). An N-terminal interaction between CBP and TBP would be supported by the homology of p300 and CBP, since p300 contacts TBP by the N-terminus (Abraham et al. 1993) (see 3.3.2.2).

A human histone acetyltransferase protein, p300/CBP-associated factor (P/CAF), has been identified and been reported to bind to p300 and CBP (Yang et al. 1996). The interaction between CBP/p300 and P/CAF is disrupted in the presence of E1A. Thus, CBP/p300 may recruit a histone acetyltransferase and thereby lead to acetylation of the histones, a phenomenon correlated with transcriptionally active chromatin (Hebbes et al. 1994).

Another interesting feature of CBP is the bromodomain (Haynes et al. 1992), a structural unit which is most likely important in contacting histones. As many of the proteins containing a bromodomain are implicated as transcriptional adapters or coactivators, it is likely that CBP acts as a coactivator. Other proteins with a bromodomain are p300, TAF250/CCG1, yeast SNF2 and yeast GCN5. The function of the bromodomain in these proteins is not clear. It is assumed that proteins acting as coactivators interact with many different proteins and the bromodomain may act as a surface for protein-protein interactions (Eckner et al. 1994).

Mutations in the CBP gene are associated with the Rubinstein-Taybi syndrome (Petrij et al. 1995). The Rubinstein-Taybi syndrome (RTS) is a syndrome that causes facial abnormalities, broad thumbs, mental retardation and broad big toes. In patients with RTS, point mutations leading to STOP-codons in the CREB-binding domain of CBP produces truncated CBP proteins. These truncated proteins have lost the ability to interact with CREB. Interestingly, the functional homologue of CBP, p300, can not overcome the defect caused by the loss of function of the mutated CBP.

### 3.3.2.1 Role of CBP in other Signaling Transduction Pathways

Originally it was thought that CBP acted as a coactivator for CREB only. Later, it turned out that CBP had the potential to contact many other transcription factors. Phosphorylation of c-Jun at Ser63 and Ser73 promotes association with CBP at the CREB-binding domain. The transcriptional activity of c-Jun is increased by phosphorylation of Ser73, which occurs through the action of the stress-activated Jun kinase (JNK). CBP enhances c-Jun-directed transcription (Arias et al. 1994). CBP was also reported to interact with several members of the nuclear receptor family, including
the retinoic acid receptors and the vitamin D receptors (Kamei et al. 1996). The interaction between the nuclear receptors and CBP occurs through a domain at the N-terminus of CBP and not through the CREB-binding domain. The protooncogene c-Myb binds to the promoters of mim-1 and c-myc and thereby activates them. The CREB-binding domain of CBP binds to c-Myb in a phosphorylation-independent manner (Dai et al. 1996). CBP binds to the region of c-Myb containing the activation domain and functions as a coactivator of c-Myb. An antisense CBP-expression vector suppresses the transcriptional activation of c-Myb. Another protein of the AP-1 family, c-Fos, binds to the CBP protein. c-Fos interacts with the C-terminal domain of CBP, where the adenoviral oncprotein 12S E1A also binds. The binding of c-Fos to CBP occurs in a phosphorylation-independent manner. CBP acts as a coactivator of c-Fos, which can be inhibited by 12S E1A (Bannister et al. 1995a). Therefore CBP acts as coactivator for both c-Fos and c-Jun. Recently, a transcription factor binding to the serum response element (SRE), the so called Sap-1a, was reported to bind to CBP. Overexpression of CBP enhanced the transcriptional activity of a reporter gene containing a SRE in the promoter, indicating that CBP acts as a transcriptional coactivator in the context of Sap-1a (Janknecht et al. 1996). Sap-1a binds to CBP in a phosphorylation-independent manner, although the transcriptional activity of Sap-1a requires phosphorylation by MAP-kinase. Thus, CBP uses different mechanisms of transcriptional activity.

Proteins encoded by the early region 1A (E1A) of human adenoviruses (Ad) regulate the expression of adenoviral genes and host cell genes. They act as repressors and activators of genes. 12S E1A is an adenoviral protein, which specifically interacts with the C-terminal domain of CBP and thereby represses transcriptional activation by CBP (Arany et al. 1995). All transcription factors so far described to bind to CBP are repressed by the 12S E1A protein. The C-terminal domain of CBP is targeted apart by 12S E1A also by the S6 kinase pp90RSK, a kinase downstream of Ras, leading to repression of transcription of cAMP-responsive genes. In addition to mediating transcriptional repression of cAMP-responsive genes, the pp90RSK-CBP complex also appears to be essential for induction of Ras-responsive genes (Nakajima et al. 1996). Simian virus 40 (SV40) large T antigen interacts with CBP/p300 at the same region where E1A interacts (Eckner et al. 1996).

Apparently, CBP is involved in many different signal transduction pathways. It serves to provide cross-talk between pathways. Since the amount of CBP is limiting in most cells, the simultaneous activation of several signal transduction pathways is prevented by CBP.
3.3.2.2 p300 is Closely Related to CBP

The protein p300 can substitute for CBP-function. Similar to CBP, p300 potentiates CREB-activated gene expression as assayed in F9 teratocarcinoma cells (Lundblad et al. 1995). 12S E1A binds to p300 and thereby inhibits its function. Thus, CBP and p300 have similar functional properties. The structure of p300 is related to CBP (Eckner et al. 1994). 12S E1A can bind to the C-terminus of p300. p300 contains a bromodomain in the middle of the protein and contacts TFIIB with the C-terminus, features which are identical to CBP. Unlike CBP, p300 interacts with TBP via its N-terminus (Abraham et al. 1993; Lee et al. 1996; Yuan et al. 1996). MyoD-dependent transactivation may involve p300, because p300 potentiates MyoD-activated transcription and MyoD can bind to p300 through a C-terminal cysteine/histidine-rich domain. MyoD-dependent gene-expression can be repressed by 12S E1A (Yuan et al. 1996). CBP/p300 has also been implicated as potential co-activator for YY-1 (Lee et al. 1995).

3.3.3 The cAMP-Response Element

The cAMP-response element CRE is a sequence found in many promoters. The CRE behaves in general like a typical enhancer: it exerts an effect independent of its orientation and position relative to heterologous promoters. However, in many enhancers, the cAMP-response is not mediated by a single CRE but by more than one cis-elements. In these cases, either multiple CREs are present or a CRE interacts with a factor bound to another cis-element. In the latter case, the second factor can provide tissue-specificity (see above). In the case of the phosphoenolpyruvate carboxykinase gene (PEPCK), transcription of the gene is strongly induced after treatment of liver, but not kidney cells, with cAMP-inducers. The liver-specific gene expression of PEPCK is ensured by cooperation among different transcription factors. The cAMP-responsiveness is provided by CREB binding to the imperfect CRE-sequence shown in Table 3-3, whereas tissue-specificity is mediated by three sites to which C/EBPα binds and by an AP-1 site. All five proteins have to bind to their respective sites for full promoter activation (Roesler et al. 1996). The tyrosine aminotransferase gene is like the PEPCK-gene; it is controlled in a liver-specific and cAMP-responsive fashion. A CRE and a HNF4-binding site located 3.6 kb upstream of transcription initiation site (see Table 3-3), have to be present for full cAMP-induction. If the HNF4-binding site is mutated to a HNF3 binding site, cooperativity is destroyed and cAMP-responsiveness is lost (Nitsch et al. 1993). A similar case is described for the human glycoprotein hormone α-subunit gene. A repeat of two consensus CREs between -146 and -111 upstream of transcription initiation site is responsible for the cAMP-response. An additional enhancer element has been proposed but not identified (Silver et al. 1987).
3.3.3.1 The Consensus CRE and the CRE-Like Site

The CRE is a palindromic sequence which is closely related to the TPA response element. In many promoters a consensus CRE sequence is present, but in some promoters it is deviated from the consensus sequence. In some promoters only half of the palindromic sequence is conserved (see Table 3-3). The importance of the deviation from the consensus CRE sequence was assessed (Nichols et al. 1992). The binding of CREB to the consensus CRE sequence (TGACGTCA) occurs independently of the phosphorylation of CREB at Ser133. If this serine is phosphorylated the binding of CREB is increased. However, if the CRE deviates from the consensus CRE or if only half of the palindromic sequence is present in an enhancer, then CREB binds only weakly when not phosphorylated. Phosphorylation at Ser133 leads to a pronounced increase in CREB binding to the imperfect CRE sites (Nichols et al. 1992). These results imply that CREB binds to consensus CREs without being phosphorylated, meaning that these CREs are constitutively occupied by CREB. The imperfect CREs in contrast are only weakly bound by non-phosphorylated CREB. Phosphorylation of CREB can then lead to binding to the imperfect sites. Thus, the imperfect CREs provide a lower basal activity but a higher relative stimulation of transcription by cAMP compared to the consensus CREs. The consensus CREs have a high basal activity and are therefore less strongly inducible by cAMP when compared to the basal activity.

<table>
<thead>
<tr>
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<tr>
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<tr>
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<td>-3407</td>
</tr>
<tr>
<td>uPA (B domain)</td>
<td>-3389</td>
</tr>
<tr>
<td>tyrosine aminotransferase</td>
<td>-3652</td>
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<td>proenkephalin</td>
<td>-98</td>
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<td>-90</td>
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<td>vasoactive intestinal peptide</td>
<td>-76</td>
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<tr>
<td>human chorionic gonadotropin</td>
<td>-143</td>
</tr>
<tr>
<td>human chorionic gonadotropin</td>
<td>-123</td>
</tr>
</tbody>
</table>

Table 3-3: cAMP-responsive elements present in genes regulated by cAMP. The CREs of different genes are compared with the consensus CRE shown in the top row. The conserved bases are underlined. The start and end positions of the CREs in their promoters are indicated. (PEPCK = phosphoenolpyruvate carboxykinase).

3.3.4 Mechanism of CREB-Dependent Gene Activation

Although pathway activated by cAMP is one of the best studied signal transduction pathways, many questions remain unanswered about the detailed mechanism of transcriptional activation (reviewed in Montminy et al. 1995). CREB is always bound to consensus CRE-sites. Phosphorylation of CREB by PKA allows the binding of CBP to CREB. CREB then undergoes a conformational change. This allosteric change is very
local and is restricted to KID. The conformation of Q2 is not affected by CBP-binding, because, if Q2 and KID are present in the cell as heterologous binding domains, they act synergistically and activate as efficiently as if they were introduced into the cell as one protein. Most likely Q2 modulates or potentiates the activating function of CBP bound to KID. In the case of non-consensus CRE-sites, the function of PKA phosphorylation on CREB is easier to understand, since phosphorylated CREB has a higher affinity to CRE-like sites than to consensus CRE-sites (Nichols et al. 1992). To summarize how CREB can lead to cAMP-dependent gene activation a model is shown in Figure 5. However, many interactions amongst the different proteins are still speculative.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Mechanism of CREB-dependent gene activation. A possible model for interactions between CREB, CBP, 12 S E1A and the basal transcription factors is shown. CREB contacts TAF\textsubscript{II}10 via Q2. The phosphorylated Ser133 is bound by CBP independent of the presence of 12S E1A. If 12S E1A is present, the basal transcription factors can not bind to the transcription initiation site. The interaction of CBP with the basal transcription factors could also occur through contacting TBP or TFIIB.}
\end{figure}
3.4 Characteristics of the Transcription Factor LFB3

3.4.1 Homeodomain Proteins: Structure and Mechanism of Transactivation

The homeodomain (HD) is a well characterized DNA-binding domain. Proteins with a homeodomain function as transcriptional regulators of many genes important during development. Homeodomain proteins play a crucial role in drosophila development. Drosophila development is profoundly affected by mutations in homeotic genes. Such mutations are capable of placing whole body parts in inappropriate locations, e.g. leading to replacement of legs with antennae or halteres with wings (Jackie et al. 1993). Homeodomain proteins were not only found in drosophila but also in vertebrates where they are similarly involved in fundamental developmental processes.

3.4.1.1 The Structure of the Homeodomain

More than 300 proteins have been identified which contain a homeodomain. The typical 60 amino acid homeodomain has been found to be surprisingly well conserved (Wright et al. 1989; Izpisua-Belmonte et al. 1992). X-ray crystallographic structure of the drosophila proteins antennapedia and engrailed and of the yeast MATa2 protein have been solved (Qian et al. 1989; Kissinger et al. 1990). A typical homeodomain consists of an amino-terminal arm and three alpha helices (reviewed in Kornberg, 1993). The N-terminal arm contacts DNA in the minor groove, whereas the third helix contacts the DNA in the major groove. The first and second helices are arranged in an antiparallel position perpendicular to the major groove of the DNA. The second and third helices have a structure similar to the prokaryotic helix-turn-helix motif, a motif utilized for DNA-recognition (Treisman et al. 1992). Thus, the specificity of the DNA-binding is provided mainly by the N-terminal arm and the third helix. However apart from the sequences in the HD additional sequences of the HD proteins have been shown to be important for the interaction with DNA and with other proteins. For example, sequences C-terminal of the HD in the ultrabithorax protein (Ubx) are crucial for determining the specific function of Ubx. Swap experiments where the Ubx-homeodomain was exchanged with the antennapedia (Antp)-homeodomain did not lead to Antp-phenotype but rather to a Ubx-phenotype, implying that sequences outside of the homeobox are crucial for Ubx-function (Chan et al. 1993). The Ubx-C-terminal part was shown to interact with the homeoprotein extradenticle (exd) (Chan et al. 1994). Moreover, in the absence of the C-terminal part, it was only necessary to change five residues in the homeodomain of Ubx to Antp-sequences to give an Antp-like function. These five residues were located partially in the N-terminal arm of the homeodomain and partially in an area where DNA-contacts are not thought to occur. Instead these
other residues, which do not contact the DNA, are located on the surface of the homeodomain, suggesting that they are able to interact with exd (Chan et al. 1993; Mann, 1995). Therefore the homeodomain is involved in protein-protein interactions apart from specific DNA-binding.

3.4.1.2 The POU-specific Domain

The homeodomain is often found in association with other conserved DNA-binding domains (Sturm et al. 1988). One such domain is the POU-specific domain, named after the three first discovered members, which contained the POU-specific domain (Pit-1, Oct-1, Oct-2 and Unc 86). The POU-specific domain is located amino-terminally to the homeodomain joined by a linker of variable length.

The POU-specific domain consists of four alpha helices which form contacts with the DNA, i.e. provides the specificity of the protein binding to the recognition site. The POU-specific domain is also important in mediating protein-protein interactions. For Oct-1 homo- and heterodimerization with other POU-homeodomain proteins has been implicated to be mediated by the POU-specific domain (Ingraham et al. 1990; Voss et al. 1991).

3.4.1.3 Transactivation by Homeodomain Proteins

The central functions of transcription factors are to activate or inhibit transcription. These functions are achieved either by a direct contact of the transcription factor with the basal transcription machinery or by indirect contact through the help of a cofactor, which acts as a bridging factor between the transcription factor and the basal transcription machinery.

In the case of homeodomain proteins several data indicate that homeodomain proteins can directly or indirectly contact the basal transcription machinery (Herr et al. 1995). In the case of the ubiquitous Oct-1 and the lymphocyte-specific Oct-2, the POU-domains can recruit a B-cell-specific cofactor, termed either OCA-B or OBF-1 (Strubin et al. 1995). OCA-B acts as a tissue- and promoter-specific coactivator and plays a determinant role in B-cell-specific gene expression (Luo et al. 1992; Luo et al. 1995).

Also cofactor-independent contacts between Oct-1 and the basal transcription complex SNAPc are described (Mittal et al. 1996). The SNAPc-complex is important in the expression of snRNP-genes, which are characterized by the presence of proximal sequence elements (PSE). The PSE are located either instead of TATA-boxes in the case of Pol II transcription-dependent U1/U2 snRNP-expression or upstream of the TATA-boxes as in the case of the U6 snRNP, which is transcribed by Pol III. The interaction
between Oct-1 and SNAPc depends strongly on one amino acid in the POU-specific part of the Oct-1 protein. Oct-1 therefore helps directly in the recruitment of the polymerase to the transcription initiation site.

Oct-1 is not only able to interact with the basal transcription machinery but can also functionally replace TBP. An interaction between Oct-1 and TFIIIB was shown by Nakshatri et al. (Nakshatri et al. 1995) on the most-likely TATA-less promoter of the lipoprotein lipase. In this promoter the octamer site appears to take over the function of the missing TATA-box. Therefore Oct-1 binds to the octamer site and recruits TFIIIB.

Similarly the homeodomain protein bicoid (BCD) forms direct contacts with the basal transcription machinery. BCD contains three activation domains, a glutamine-rich, an alanine-rich and an acidic activation domain. BCD can bind selectively to two different subunits of TFIIID, TAF\textsubscript{IIID}110 and TAF\textsubscript{IIID}60 (Hoey et al. 1993; Weinzierl et al. 1993). BCD contacts both TAFs via different transactivation domains. The TAF\textsubscript{IIID}110 is contacted by the glutamine-rich domain of BCD, whereas the alanine-rich domain of BCD interacts with TAF\textsubscript{IIID}60 (Sauer et al. 1995). Both domains have the potential to activate transcription independently. For synergistic activation both domains are required.

These data corroborate the idea that homeodomain proteins can directly interact with TAFs but can also use cofactors to mediate the contact with the TAFs.

3.4.2 The Homeodomain Family of LFB1

3.4.2.1 Introduction to LFB1/3, Evolution and Function

LFB1, liver-factor B1, was one of the first identified liver-enriched transcription factors. It is also called HNF1 (hepatocyte nuclear factor 1), HNF1\(\alpha\) or rarely HP1 or APF (see Cereghini et al. 1990). Several liver genes, such as albumin, fibrinogen and aldolase B were identified which are regulated by LFB1. These gene products are involved in various metabolic functions, (reviewed in Mendel et al. 1991a; Cereghini, 1996a). By homology screening, a protein related to LFB1 was identified and named LFB3, HNF1\(\beta\) or vHNF1 (variant HNF1). LFB1 and 3 bind to the same palindromic recognition site GGTTAATNATTAAC(A/C). The upstream moiety of the binding site is more conserved than the downstream moiety. The GTTA sequence is the most highly conserved region (Frain et al. 1989; Tronche et al. 1992). LFB1 and LFB3 can both bind to this sequence either as homo- or heterodimers.

LFB1 was originally purified from rat liver nuclear extracts and was shown to be heavily glycosylated with an apparent molecular weight of 87-93 kDa (Frain et al. 1989;
Lichtsteiner et al. 1989; Chouard et al. 1990). The function of the purified protein was assayed in spleen nuclear extracts, where the albumin promoter is inactive because of the absence of LFB1. Addition of the purified protein to spleen nuclear extracts stimulates transcription from the albumin promoter (Lichtsteiner et al. 1989).

LFB1 has been cloned from mouse (Kuo et al. 1990), rat (Chouard et al. 1990), human (Bach et al. 1990), chicken (Horlein et al. 1993), hamster (Emens et al. 1992), Xenopus (Bartkowski et al. 1993) and salmon (Deryckere et al. 1995).

The LFB3 cDNA has been isolated from pig (Menoud et al. 1993), mouse (De Simone et al. 1991), rat (Rey-Campos et al. 1991), human (Bach et al. 1991) and Xenopus (Demartis et al. 1994). Of all these species, salmon is phylogenetically the most distant. A sequence comparison of all these proteins with the salmon LFB1 is shown in Table 3-4. The salmon LFB1 displays homology to both proteins, LFB3 and LFB1 almost equally, i.e. it is 56% homologous to human LFB1 and 49% to human LFB3. Therefore the salmon LFB1 is almost as closely related to human LFB3 as it is to human LFB1. Identification of a salmon homologue of LFB3 has so far failed, possibly due to the complete absence of LFB3 in salmon (Deryckere et al. 1995). In evolution there might exist a common ancestor of LFB1 and 3 and the two different proteins could then have evolved after the fishes diverged from the lineage leading to mammals (Deryckere et al. 1995). In Figure 6 a phylogenetic tree of the LFB1/3 proteins is displayed.

LFB1 is expressed in various tissues in the adult, being highly expressed in liver, kidney, intestine and pancreas (Baumhueter et al. 1990; Blumenfeld et al. 1991; Lazzaro et al. 1992; Bartkowski et al. 1993). Mice deficient in LFB1 were born a little bit smaller than normal mice (Pontoglio et al. 1996) and they died around weaning. Although the high presence of LFB1 in the liver would should cause these mice to die from a lack of certain liver-specific proteins, they actually die from renal failure after a progressive wasting syndrome. In these mice the liver is enlarged. Several hepatic genes, such as albumin and α1-antitrypsin are expressed at abnormally low levels. The gene encoding phenylalanine hydroxylase is completely silent giving rise to phenylketonuria. The lack of LFB1 in kidney, where LFB1 is expressed in the proximal and distal tubule, results in massive urinary glucose loss, leading to energy and water wasting. The phenotype of these mice is similar to the human renal Fanconi-syndrome, a hereditary disease characterized by glycosuria, phosphaturia, proteinuria, hypercalcuria and bone diseases (Friedman et al. 1978). The general cause of this syndrome may be a defect in the proximal and distal tubular function of the nephron. A defect in these renal areas leads either to a decreased reabsorption from the proximal tubule or to an augmented...
permeability of the membrane in the distal tubules leading to an increase in glucose, phosphate and amino acid concentration in the urine (Bergeron et al. 1976). The loss of glucose finally leads to the death of these mice. Defects on different levels in the generation of urine can lead to Fanconi-syndrome. Because the primary cause of Fanconi-syndrome is still unknown, mice lacking LFB1 may be a helpful model for the study of this disease (Pontoglio et al. 1996).

![Phylogenetic tree of the LFB-family.](image)

**Figure 6: Phylogenetic tree of the LFB-family.** The salmon LFB1 protein is the most distant member from the LFB1/3-protein family.

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</tbody>
</table>

**Table 3-4: Homology of salmon LFB1-protein with other members of the LFB1-family.** The salmon LFB1 is almost as closely related to LFB1 as it is to LFB3. For the comparison the HNF1-A isoforms were used. The length of each protein is indicated, together with the number of amino acids that align with the salmon LFB1 amino acids.

### 3.4.2.2 The Structure of LFB1/3

In line with the fact that LFB1 and LFB3 diverged rather late in evolution, their structures are very similar, the major difference being in the C-terminal part of the proteins, as shown in Figure 7. The overall similarity between LFB1 and LFB3 is 58% (Mendel et al. 1991b). Several putative glycosylation sites are found in LFB1/3. The
general structure of both proteins is a tripartite DNA-binding domain located at the N-terminus and a transactivation domain at the C-terminus (Nicosia et al. 1990). The main feature of the DNA-binding domain of LFB1/3 is the atypical homeodomain, which is about 95% homologous in LFB1 and LFB3. Thus, LFB1/3 belong to the homeodomain family, but they are unique among them, since they contain an atypical homeodomain (Leiting et al. 1993). The structure of the helices is the same as that found in Antp or engrailed (Ceska et al. 1993). However, between the second and third helix of LFB1/3, 21 amino acids are inserted. These additional amino acids form an extended loop between helix 2 and 3, which could participate in dimer formation (Finney, 1990). The extended loop contacts residues in the loop between helix 1 and 2, does not contact the DNA and is relatively flexible. The extended loop may change conformation upon dimer formation, inducing a conformational change of the DNA, most likely DNA bending. Another possibility is that the LFB1/3 homo- or heterodimer-subunits are rearranged relative to each other by the conformational change of the extended loop (Ceska et al. 1993).

**Figure 7: Comparison of LFB1 and LFB3 isoforms.** The N-terminal region of all these proteins is highly homologous and forms a tripartite DNA-binding domain. At the C-terminal area the activation domain is localized, where isoforms are mostly divergent. AD = Activation domain, DD = dimerization domain, POU = POU-specific domain, Pro = proline-rich, Glu = Glutamine-rich, Ser = Serine-rich AD.

Apart from the homeodomain, the DNA-binding domain consists of a highly conserved dimerization domain of 31 amino acids at the N-terminal end of both LFB1 and 3 (Nicosia et al. 1990). This dimerization domain is unique among homeodomain proteins, in that it allows LFB1/3 to exist as dimer before binding to DNA. The dimerization domain has weak homology to the myosin heavy chain (Nicosia et al. 1990a; Mendel et al. 1991a). The protein DcoH has been reported to bind to this dimerization domain, as described below. Between the dimerization domain and the homeobox, a POU-domain-like element is localized. This domain is quite diverged from the common four-helical structure of POU-domains but is necessary for specific DNA-
binding. In LFB1/3, the two N-terminal helices of the usual four helices are only partially present (Tomei et al. 1992).

The carboxy-terminal region of LFB1/3 makes up the transactivation domain (Sourdive et al. 1993; Toniatti et al. 1993). This part of LFB1 has only 47% homology with LFB3. In the LFB1 C-terminal of the homeodomain, three distinct activation domains (AD) have been characterized (Toniatti et al. 1993). The first activation domain (ADI), between amino acids 546 and 628, has the highest activation potential and is serine-rich. The second activation domain (ADII), which is proline-rich and lies between 281 and 318, is little, if at all, able to activate the transcription machinery. An additional glutamine/proline-rich activation domain (ADIII) between amino acids 440 and 506 has been identified. However, the transactivation potential of the individual activation domains varies depending on the experimental setup.

A nuclear localization inhibiting signal is found in the C-terminus. The full length protein (628 amino acids long) and a truncated protein with only the first 289 residues is localized in the nucleus, whereas deletion mutants containing the first 348 or 416 amino acids do not enter the nucleus (Sourdive et al. 1993).

In contrast to LFB1, LFB3 has a shorter transactivation domain, which is homologous to the ADIII of LFB1. Because of the short transactivation domain, it has been speculated that LFB3 is a repressor of LFB1, however LFB3 turned out to be an activating transcription factor (De Simone et al. 1991; Rey-Campos et al. 1991).

Many transcription factors are known to be expressed as families of protein isoforms encoded by alternative spliced mRNAs. The isoforms of some factors are expressed at a constant ratio throughout development, but in many cases differential splicing is observed according to developmental stages and tissue-type. Multiple isoforms of LFB1 and LFB3 exist which are generated by alternative splicing and the use of alternative polyadenylation sites. All these isoforms differ in their carboxy-terminal regions, suggesting that their transactivation potentials are different. The two LFB1 isoforms are called HNFl-B and HNFl-C (Bach et al. 1993). HNFl-B and -C proteins are stronger transactivation activators than HNFl-A, as the previously termed LFB1 was renamed. The possibility of acquiring different activation domains implies that subtle changes in the activation potential are accompanied by physiological alterations in target gene expression levels. Two additional LFB3-isoforms are described: vHNFl-B and vHNFl-C (the previously described LFB3 has been renamed to vHNFl-A (Rey-Campos et al. 1991; Bach et al. 1993). vHNFl-B lacks about 20 amino acids between the POU-like domain and the homeodomain. In vHNFl-C, however, a large region of the C-terminal transactivation domain is deleted. vHNFl-C is a potent repressor of LFB1
These isoforms are differently expressed in fetal and adult liver, kidney, thymus and intestine, suggesting that they fulfill different roles during development (Bach et al. 1993). Therefore, the activation domains of LFB1 and LFB3 are organized in modular structures, allowing different genes to be differently regulated according to the combination of LFB1/3 heterodimers expressed in a certain cell in a certain tissue or differentiation state.

In the present work, pig LFB3 was studied, which corresponds to the vHNF1-A isoform (Menoud et al. 1993). Computational analysis using the Wisconsin Package revealed several putative phosphorylation sites by protein kinase C (PKC) and casein kinase II (CK-2). The activities of both proteins is unaffected by cyclic nucleotides. No protein kinase A phosphorylation site was detected.

3.4.2.3 DcoH, a Cofactor of LFB1/3

So far only one cofactor has been described for LFB1/3. This factor, termed DcoH (dimerization cofactor for HNF1-α), does not bind to DNA itself but binds to the dimerization domain of LFB1. The 11 kDa protein is identical to pterin-4a-carbinolamine dehydratase (PCD) (Citron et al. 1992). The PCD-enzymatic activity is required for the first step in the synthesis of tetrahydrobiopterin, an essential cofactor in the synthesis of amino acids, and its derivatives, including tyrosine and serotonin.

The tetrameric complex of LFB1-dimer and DcoH-dimer has an increased transcriptional activity, but does not stimulate transcription in the context of the GAL4 DNA-binding domain. DcoH is expressed highest in liver and kidney, and at lower levels in intestine, stomach and lung (Mendel et al. 1991c). The structure of DcoH was solved at 2.7 Å resolution. The functional DcoH protein forms a homotetramer, with a structure which is partially TBP-like. Therefore, it could be that DcoH binds to DNA (Ficner et al. 1995). The expression of DcoH during development was studied in Xenopus, in whose eggs DcoH is present as maternal factor. Rat and Xenopus DcoH are 85% homologous (Pogge von Strandmann et al. 1995). In Xenopus, the amount of DcoH increases drastically following neurulation at the timepoint where the liver and the prospective kidneys are formed. The DcoH gene is localized in mice and humans on chromosome 10 (Milatovich et al. 1993). The effects caused by DcoH are the subject of controversy, because some laboratories, including ours, could not reproduce the results obtained by Mendel et al. (1991c). Currently, mice deficient in DcoH are generated to characterize the phenotype (J. H. Bayle, personal communication).
Although LFBl and LFB3 are highly homologous, the expression patterns for both proteins are different. LFB3 has a broader distribution pattern than LFBl, therefore LFB3 is present in some tissues where LFBl is absent (De Simone et al. 1991). These tissues include the lung, the stomach and the thymus. Both proteins are expressed in the kidneys, the liver, the pancreas and the intestine as mentioned above. In the kidney, LFBl is expressed in the proximal and distal tubule, LFB3 in contrast is also expressed in the collecting tubule. The promoter of the rat LFBl has been cloned to a length of 3.5 kb upstream of the transcription initiation site (Piaggio et al. 1994). It contains a CCAAT-signal around 130 bp upstream of the transcription initiation site and lacks any TGACG-element. The LFBl promoter activity is down-regulated by the addition of LFBl protein. In this way, LFBl acts as a repressor of its own transcription. However it has not been possible to show binding of LFBl to its own promoter.

LFB3 precedes the expression of LFBl during development. At the onset of liver development, LFB3 is already detectable, in contrast to LFBl which is expressed during organogenesis of the liver. In general, LFBl is expressed only in fully differentiated cells, whereas LFB3 is also expressed in dedifferentiated cells (Ott et al. 1991). LFB3 apparently participates in deciding cell fate, whereas LFBl is only required to maintain the differentiated state (Bartkowski et al. 1993). However, LFB3 is constantly expressed in certain differentiated cell-types. The LFB3 promoter was cloned and five sites of DNA-protein interactions were found within the first 260 bp upstream of the transcription initiation site. These sites are DR1-motifs and Oct-motifs. DR-1 elements are recognition sequences for members of the steroid hormone receptor superfamily (Tsai et al. 1994). Consequently, the LFB3 promoter fused to a reporter gene can be stimulated by Coup-TF1/Ear3 and Coup-TFII/Arp1 but, unlike HNF1, not by HNF4 (Power et al. 1996).

Embryonal carcinoma cells are a valuable tool for studying differentiation events in embryos before implantation because they resemble pluripotent cells, which can be induced to differentiate into certain cell types when the cells are treated with various chemical inducers. The mouse teratocarcinoma cell line F9 differentiates into parietal endoderm cells upon treatment with retinoic acid (RA) and Bt2-cAMP (Strickland et al. 1980). RA alone induces differentiation of F9 cells into visceral endoderm (Hogan et al. 1981; Strickland et al. 1987). Interestingly, F9 cells can also be induced to differentiate using okadaic acid (Nishina et al. 1995). Both cells are derived from a common precursor cell in the mouse embryo, known as primary endoderm. Both cell types differ in morphology and biochemical properties. The F9 cells can also be induced into nerve-
like cells with adrenergic characteristics (Liesi et al. 1983). Upon differentiation, a variety of proteins become expressed which are absent in undifferentiated cells (UF9). These proteins include α-fetoprotein (Hogan et al. 1981), c-Fos, c-Jun, junB, tPA (Nishina et al. 1995) and laminin B1 (Nishina et al. 1995). UF9 cells do not express LFB1 nor LFB3. Upon differentiation using RA, F9 cells start to express LFB3 (De Simone et al. 1991), low levels of aberrantly expressed LFB1 (Cereghini et al. 1992) and HNF4, supporting the above finding that LFB3 is expressed early in mouse development.

3.4.3 Liver-specific Gene Expression

3.4.3.1 The cis-and trans-acting Factors

One of the best characterized models for tissue-specific gene expression is the transcriptional control of the liver-restricted albumin gene. The regulation of liver-specific gene transcription has been intensively studied (reviewed in Cereghini, 1996). The analysis of the albumin gene promoter, together with the study of other liver-specific promoters and enhancers, has led to the discovery of four families of liver enriched transcription factors. These families include the variant homeodomain containing family HNF1 (Mendel et al. 1991b), the CCAAT/enhancer-binding protein family, the winged helix family HNF3 and the members of the nuclear receptor superfamily HNF4.

The study of liver-specific gene expression has revealed some basic principles which also apply to other cell differentiation systems. The cis regulatory sites are arranged in modular structure where cell-restricted and ubiquitous transcription factors usually bind. None of the cell-restricted transcription factors are exclusively expressed in liver, suggesting that a unique set of transcription factors is present in a certain cell-type which synergistically regulates, either positively or negatively, a certain subset of genes, which then define the phenotype of the cell. Therefore, the combination of several distinct classes of transcription factors provides the basis for a tissue-restricted and synergetic gene expression, with the possibility that a large number of factors can participate in the control at this gene promoter. Another way that cell-specific gene expression is achieved is by transcription factors which bind to the same or overlapping recognition sites regulating gene expression by competing with each other at theses sites. The ability of transcription factors to form homo- and heterodimers greatly enlarges the number of potential factors binding to a certain site. However, it is also obvious that other means exist which control cell-specific gene expression such as the
organization of the chromatin structure, protein-modifications, and the presence of cell-specific cofactors and coactivators.

3.4.3.2 A Network of Factors Coordinates the Development of Differentiated Hepatocytes

The endoderm of mammals gives rise to the liver, thymus, pancreas and certain other tissues. During the development of the liver, a network of transcription factors regulates specific transcription in hepatocytes (reviewed in Tronche et al. 1992; Cereghini, 1996).

The transcription factor HNF3 is the first to be expressed in the endoderm during development. The HNF3 family is similar to forkhead and binds DNA as a monomer. The DNA binding motif is a helix-turn-helix variant termed "winged helix". HNF3 is expressed in the adult in the endoderm derived organs including liver, lung, brain, kidney, thymus and pancreas.

C/EBP, the CCAAT/ enhancer binding protein, exists in four different isoforms, called C/EBP α, β, γ and δ. They form homo- and heterodimers in vitro via their basic leucine zipper-motif. C/EBPα is present in differentiated hepatocytes and adipocytes. In contrast, C/EBPβ, also called LAP, IL6 or IL6DAP, is almost ubiquitous, specially highly expressed in liver and lungs (Descombes et al. 1990; Descombes et al. 1991). C/EBPβ is modulated by PKA, PKC and the MAP-kinase. C/EBPβ is activated by a very unusual mechanism. The phosphorylation of C/EBPβ results in the inactivation of an inhibitory domain, i.e. it is activated by derepression through dephosphorylation.

HNF4 belongs to the nuclear receptor superfamily and binds to DNA as a dimer with its zinc finger. Since no specific ligand for HNF4 has been identified, it is an orphan receptor. HNF4 is closely related to CoupTFI/Ear3 and CoupTFII, also termed Arp1. CoupTF-factors act as negative regulators of HNF4 by competing directly with HNF4 at the DNA-binding site, which is a repeat of AGGTCA. HNF4 is expressed in the adult liver, intestine and kidney. Like LFB3, HNF4 is expressed before LFB1 during development (Cereghini et al. 1992; Cereghini, 1996). In fact, HNF4 acts as a positive regulator for LFB1 expression (Tian et al. 1991; Kuo et al. 1992).

In the development of the liver, a whole network of factors exist. The first factors expressed are HNF3-proteins, followed by LFB3 and HNF4. LFB1 appears later, at the time of liver organogenesis. C/EBP is expressed late in the development suggesting that it does not participate in the early development, although HNF3 contains a weak binding site for C/EBP.
HNF3 may be induced by RA and its expression is then reinforced by an autoregulatory loop (Pani et al. 1992). HNF4 and, to a lesser extent, HNF3 and eventually LFB3 induce LFB1 (Tian et al. 1991; Kuo et al. 1992). LFB1, in contrast, negatively regulates its own gene and the HNF4 gene (Kritis et al. 1993; Piaggio et al. 1994; Zhong et al. 1994). Data on the autoregulation of LFB1 are controversial. Experiments performed in different cell lines have not shown the same results. In experiments performed in HeLa-cells, a construct containing the area of -3500 to +88 of the LFB1-promoter fused to a reporter gene was induced by cotransfection of an HNF4-expression vector, and this induction by HNF4 was repressed by cotransfection of LFB1 (Piaggio et al. 1994). An almost identical result has been obtained from HepG2-cells, where only the region from -242 to +44 of the rat LFB1 promoter was used (Kritis et al. 1993). An opposite effect of LFB1 on its own promoter has been reported, where LFB1 could weakly stimulate the rat LFB1-promoter from -561 to +58 in P19 cells (Miura et al. 1993). Unlike the LFB1-expression, the LFB3-expression does not depend on HNF4 but rather on retinoic acid and on Coup/TF, allowing expression of LFB3 earlier than LFB1 during development (Cereghini et al. 1992).
3.5 The Plasminogen Activators

Plasminogen activators (PAs) are extracellular proteases which convert plasminogen into plasmin. Two different PAs exist, called tissue-type (tPA) and urokinase-type (uPA) PAs, respectively. Plasmin itself is also a protease which has, in contrast to the PAs, a broad trypsin-like substrate-specificity. Through the activation of plasminogen to plasmin, PAs play a key role in many biological processes involving extracellular proteolysis. These include among them tissue remodeling, cell migration, fibrinolysis and normal and pathologic invasive cell growth (reviewed in Saksela et al. 1988).

3.5.1 The Components of the Plasminogen Activation System

3.5.1.1 Plasminogen and Plasmin

Since PAs exert their biological effects by converting plasminogen into plasmin, it is important for the understanding of their function to focus on the substrates of plasmin. The “classical” substrates of plasmin are fibrin and fibrinogen (Liotta et al. 1981). Other substrates are blood clotting factors such as factor XII, proteins in the extracellular matrix and procollagenase. Plasmin is therefore a key component in dissolution of fibrin accumulations and is critical in the maintenance of hemostatic balance (Plow et al. 1995). Plasmin also acts in concert with other proteases, which play a role in the degradation of the extracellular matrix in the context of physiological and pathological tissue remodeling and cell migration events, such as ovulation, trophoblast invasion, postlactational mammary gland involution, wound healing, angiogenesis and tumor cell invasion (Folkman et al. 1992; Tuszynski et al. 1996).

Plasminogen is converted into plasmin by a single proteolytic cleavage at Arg560 (Robbins et al. 1967). Plasminogen is present in most if not all extracellular fluids. It is synthesized in the liver, testis and possibly in human epidermal cells. Plasminogen binds to various proteins including vitronectin, thrombospondin, laminin, fibronectin and fibrin (Lucas et al. 1983; Chain et al. 1991; Mosher et al. 1992; Stephens et al. 1994; Kost et al. 1996).

Mice rendered plasminogen deficient by gene targeting complete embryonic development, survive to adulthood and are capable of reproduction, although they develop multiple spontaneous thrombotic lesions leading to severe organ damage and high mortality rate at an early age (Bugge et al. 1995). These findings indicate that plasminogen is not strictly required for tissue remodeling processes associated with development, growth and reproduction, possibly because of an overlap between plasmin and other extracellular proteases. Apparently these other extracellular proteases are
sufficient to compensate, at least partially, for the loss of plasmin-mediated proteolysis. However in a recent study by Romer et al. (1996), mice deficient in plasminogen showed severely impaired skin repair during wound closure, which demonstrates that plasmin is required for normal wound repair. This result further supports the idea that plasminogen participates in many processes where the extracellular matrix is degraded.

Apart from the above functions, plasmin is also involved in regulatory functions. It can activate enzymes and molecules involved in signal transduction. Plasmin activates latent growth factor TGF-β complexes or releases matrix-bound growth factors, such as basic fibroblast growth factor (bFGF) through the activation of the latent form of collagenase. These growth factors can have a positive effect on PA-production (Laiho et al. 1986; Keski-Oja et al. 1988). Thus, plasmin can potentiate its own effect by activating signal transduction pathways leading to the formation of plasmin. Another mechanism resulting in a potentiation of the plasmin activity is achieved by the ability of plasmin to cleave protease zymogens, most remarkably the zymogen pro-uPA and some metalloproteases. The generation of uPA by plasmin leads to a very potent positive feedback regulation culminating in the generation of many active plasmin molecules. In this way plasmin, the PAs and the other factors involved in this system can lead to an amplification and extension of the degradative process (reviewed in Plow et al. 1995).

On the other hand, an uncontrolled activation of plasmin would have deleterious effects. Specific mechanisms must therefore exist to control initiation, progression, localization and termination of the system. Since plasminogen is ubiquitously expressed in the body, the localized activation of plasminogen is achieved by the localized activation of the plasminogen activators.

3.5.1.2 The Plasminogen Activators

In the body two types of PAs are present: the tissue-type PA (tPA) and the urokinase-type PA (uPA). These PAs and plasmin are all members of the serine protease family which includes chymotrypsin, trypsin, elastase and enzymes of the complement cascade. Although the amino acid sequences of the two human PAs share only 40% similarity and they are immunologically distinct, the enzymes are very similar in their overall structures.

An immature form of uPA is secreted as a 52 kDa single chain glycoprotein (pro-uPA). Pro-uPA consists of four distinct domains: a growth factor domain, which mediates receptor binding (see 3.5.2.2); a kringle domain, which is homologous to those in plasminogen and tPA; the connecting peptide and the catalytic region. After secretion,
pro-uPA is avidly bound by the surface uPA-receptor. Pro-uPA is converted into active uPA by plasmin. The activation of pro-uPA by plasmin is enhanced when both proteins are bound to their cell-surface receptors. Cell-surface receptors for uPA are necessary since these receptors concentrate uPA at the cell-surface, because uPA occurs at very low levels in normal human plasma.

uPA is present at much higher concentrations in urine than in human plasma as it is produced in the renal tubular epithelium. Urine has been intensively used as the source for the purification of uPA. The supernatants of tumor cell cultures can serve as alternative sources for uPA.

The central role of tPA as a thrombolytic agent is widely accepted and it is often used clinically. tPA has a high specificity for fibrin and has an enhanced activity when associated with fibrin. In this way, a localized activation of tPA is ensured. However, tPA may also be involved in a plethora of other physiological and pathological functions including metastasis, ovulation and angiogenesis. tPA is produced by vascular endothelial cells after their stimulation with various agents including hormones, cytokines and proteolytic enzymes. It is thought that tPA is responsible mainly for the maintenance of the homeostasis by the dissolution of fibrin clots whereas the role of uPA in thrombolysis is less well defined. uPA lacks affinity for fibrin and is rather considered to be involved in extracellular proteolytic activities such as the degradation of basal membranes and tissue remodeling. Hence uPA may play a broader biological role than tPA.

Knock-out mice where either the tPA- or the uPA-gene was deleted showed only a few dysfunctions indicating that both PAs may share many functions (Carmeliet et al. 1994). uPA-deficient mice developed spontaneous fibrin deposits and increased thrombosis, whereas tPA-deficient mice revealed a higher incidence of thrombosis and a reduced thrombolytic potential. Only the deletion of both PA-genes in the same mice led to severe impairments, such as reduced fertility and shortened life-span.

3.5.1.3 Plasminogen Activator Inhibitors

To avoid uncontrolled plasminogen activation, specific inhibitors effectively inactivate PAs. To date four inhibitors have been described: PAI-1, PAI-2, PAI-3 and PN-1. All these inhibitors belong to the serine proteinase inhibitor family (serpin) (Lawrence et al. 1990). All have an arginine in the reactive center and are therefore classified as members of the arg-serpin subgroup. This class of inhibitors inactivates the serine proteinase by the formation of a covalent bond between the arginine and the reactive center of the proteinase.
The first described inhibitor, the Plasminogen Activator Inhibitor 1 (PAI-1), was detected in endothelial cells. PAI-1 is produced by vascular endothelium and platelets, as well as hepatocytes, fibroblasts, placental trophoblasts and cultured tumor cells. PAI-1 is considered to be principally involved in the regulation of vascular fibrinolysis. It is found in normal human plasma and in increased levels during sepsis and pregnancy and is used in the prognosis of metastases (Pappot et al. 1995). Stimulators of PAI-1 secretion include TNF-α, IL-1, TGF-β, EGF and glucocorticoids (Lund et al. 1987b; Keski-Oja et al. 1988b; Medcalf et al. 1988b; Medcalf et al. 1988b). PAI-1 is regulated by many effectors that also affect PA expression. For example IL-1 induces both PA- and PAI-1-expression. The concomitant stimulation of PA and its inhibitor leads to a controlled localized degradation of the extracellular matrix. PAI-1, like uPA, is bound to the cell-surface by vitronectin (Stephens et al. 1993). This interaction between PAI-1 and vitronectin stabilizes PAI-1, which otherwise rapidly loses activity when secreted.

PAI-1 reacts with uPA, tPA and activated protein C. PAI-1 reacts with these proteins by forming an equimolar and inactive covalent complex. This inactivation reaction is slower but still functional when tPA is bound to fibrin, and also occurs when uPA is bound to the cell-surface uPA receptor. Thus PAI-1 is able to inhibit uPA mediated extracellular matrix degradation.

PAI-2 was first called the placental PAI because it was originally described in extracts prepared from placenta. PAI-2 is also expressed by cells of the macrophage lineage (Bachmann, 1995) and is primarily an inhibitor of uPA. The binding of PAI-2 to tPA is much slower than PAI-1, especially when tPA is bound to fibrin. PAI-2 is not detectable in normal plasma or during sepsis, but it appears during pregnancy due to secretion from the placental trophoblastic epithelium. Unlike PAI-1, secreted PAI-2 does not spontaneously lose activity, and therefore remains active in solution; no binding proteins for PAI-2 have been detected in the pericellular matrix. Clinical studies have identified PAI-2 as a indicator in the prognosis of a number of carcinomas, such as lung carcinomas (Nagayama et al. 1994) where it is thought to inhibit cell-surface uPA-mediated proteolysis and thereby limit the metastatic potential of the tumor. Recent data have also highlighted a previously unexpected role for PAI-2 as a marker in apoptosis and an inhibitor for TNF-mediated cytotoxicity (Kumar et al. 1991).

PAI-3 was isolated from human urine. This molecule turned out to be identical to the protein C inactivator found in plasma. It inhibits both uPA and tPA, but with a slower rate than the other inhibitors.
Protease nexin-1 (PN-1) is a less specific inhibitor than PAI-1 or PAI-2; it also inactivates trypsin, plasmin and extremely rapidly thrombin. It was isolated from cultured fibroblasts but it is also produced by other cultured cell lines.

The individual roles of the above inhibitors in PA regulation are not clear. Each inhibitor most likely has a separate and specific function as shown by the differences in their biological properties. Apparently a whole network exists which controls the generation of free plasmin.

3.5.2 Assembly of the Plasminogen System on Cell Surfaces

The binding of the components of the plasminogen system to surfaces is of crucial importance in controlling the function of this system (reviewed in Plow et al. 1995). Both the fibrin surface and the cell surface exert similar regulatory functions, because plasmin and plasminogen bind to both surfaces. The surface plays an important role because plasminogen activation is enhanced as a consequence of surface binding and its proteolytic activity is protected from inhibitors that would inactivate free plasmin. The physiological relevance of the cell-surface associated proteolytic activity of plasmin is apparent in cell migratory responses, where plasmin helps to digest the extracellular matrix and in this way helps cells to dissolve their attachments.

3.5.2.1 Activation of the Plasminogen System on Cell Surfaces

Not only plasmin but also many other components of the plasminogen system, including uPA and tPA, can bind to cell surfaces. These receptor-bound PAs, either tPA or uPA, can activate the cell-bound plasminogen into plasmin. Free plasminogen is far less efficiently activated. Plasmin in contrast can activate receptor-bound pro-uPA to the more active uPA. This leads to an amplification of PA activity and plasmin production. Another mechanism of amplification of PA activity is achieved by the ability of plasmin to activate latent plasminogen binding sites through its ability to create carboxy-terminal lysine residues after proteolytic digestion. An effect leading to the formation of new binding sites for plasmin and hence to the recruitment of more plasmin to the cell-surface. As plasmin dissociates from the surface, its activity is rapidly inhibited by α2-antiplasmin.

3.5.2.2 Plasminogen Receptors and uPA-Receptors

The binding of plasmin to the cell-surface is crucial for its activity and therefore specific receptors for plasmin exist. Plasminogen receptors have three main characteristics: they are ubiquitously distributed; have a low affinity and are present in
high density. The low affinity with a $K_D$ of about 0.1-2 μM is compensated for by the presence of the receptors at high concentrations on the surface (Miles et al. 1988). The low affinity leads to a high turnover rate of plasmin bound to the surface and is most likely essential to limit excessive proteolysis.

As mentioned above apart from plasminogen receptors, specific binding sites for uPA do exist (Blasi, 1988). The uPA-receptors (uPAR) were first described in monocytes (Vassalli et al. 1985) and monocyte-like U937 cells (Stoppelli et al. 1985) and subsequently in many types of cultured cells, including a wide variety of neoplastic origin (Plow et al. 1986; Nielsen et al. 1988). The function of uPAR is to recruit not only uPA to the cell surface but also to locate uPA in discrete areas of the cell membrane. uPA- and uPAR-immunoreactivity was found in certain adherent cells at focal adhesion sites, colocalizing with the intracellular protein vinculin, a specific marker for these contact sites, as well as in areas of cell-cell contact (Pollanen et al. 1988; Myohanen et al. 1993).

uPAR expression depends on the condition of the cell, since metastatic cells have an increased number of uPARs. Therefore uPAR is a crucial component of the plasminogen system, which controls the distribution of uPA activity over the cell. Thus, uPAR concentrates uPA-activity to only special regions of the cell-surface.
3.6 Regulation of uPA Gene Expression in LLC-PK₁ Cells

LLC-PK₁ is a cell line derived from pig kidney epithelia (Hull et al. 1976; Handler et al. 1991). These cells express several properties of the renal proximal tubule, *pars recta* (Gstraunthaler, 1988). Similar to cells from the mammalian proximal tubule, LLC-PK₁ cells have Na⁺-dependent hexose transporters (Rabito, 1981) and Na⁺-coupled phosphate transporters (Biber et al. 1983). The expression of these transporters is altered by changes in the concentration of glucose and inorganic phosphate in the culture medium.

Gene-regulation is achieved on many levels: on the transcriptional; posttranscriptional and posttranslational levels. Similarly, the uPA gene is also controlled on different levels. In untreated LLC-PK₁ cells, only a small amount of uPA is detectable in the culture medium. However, upon treatment of the cells with various agents, the concentration of PA activity in the supernatant of culture medium increases with time. In many cases increased uPA protein concentrations were paralleled by increased uPA mRNA levels indicating that the transcriptional regulation of the uPA gene and the control of its mRNA stability are essential to the overall regulation of uPA gene expression.

In LLC-PK₁ cells the uPA gene is induced by 12-0-tetradecanoylphorbol-13-acetate (TPA) (Lee et al. 1993), the protein phosphatase 1/2A inhibitor okadaic acid (Nagamine et al. 1991b; Lee et al. 1994b), cytoskeletal reorganization (Botteri et al. 1990; Lee et al. 1993) and agents that raise intracellular cAMP levels (von der Ahe et al. 1990). Therefore the uPA gene is regulated by many different signaling transduction pathways (reviewed in Besser et al. 1996). To understand how these signal transduction pathways affect the expression of uPA, the availability of uPA cDNA clones from pig (Cassady et al. 1991), mouse and human libraries (Riccio et al. 1985) has enabled the research to locate sites involved in the uPA gene induction in different cell lines. Interestingly, the highest homologies between the promoters were found in the area around 2.5 kb upstream of the transcription initiation site, whereas the more upstream or downstream promoter parts were less well conserved between mouse and pig. For the human promoter the upstream sequences are not yet known. The cAMP-regulation of the uPA gene is best studied so far in LLC-PK₁ cells.

3.6.1 The LLC-PK₁-Cells as a Model System for Studying Transcriptional Activation by the cAMP-Dependent Protein Kinase Pathway

The LLC-PK₁ cells became an important system for the study of the cAMP-dependent protein kinase pathway due to the discovery that plasminogen activity in the
culture medium was increased after treatment with calcitonin and vasopressin and this was correlated with an increase in intracellular cAMP concentrations (Dayer et al. 1981).

In LLC-PK1 cells, the hormone vasopressin acts through a V2 receptor to stimulate the production of cAMP. Vasopressin (VP) is secreted by the posterior pituitary and this secretion is regulated by osmoreceptors in the brain. An increase in the osmotic pressure of the plasma stimulates the secretion of VP. The function of VP is to reduce the osmotic pressure of the plasma for the sake of homeostasis. The main target of VP action is the kidney. The function of VP on the kidney is best explained by considering the two extremes: absence of VP or maximal stimulation by VP. In the absence of vasopressin the kidney produces a large volume of urine with an osmotic pressure lower than that of plasma. Under these conditions the kidney is excreting water in excess. The resulting hypo-osmotic urine tends to increase the osmotic pressure of plasma. Under maximal stimulation by vasopressin, the kidney produces a small volume of hyperosmotic urine; the osmotic pressure of the urine is greater than that of plasma. Thus, VP exerts a dual effect on the kidney: it controls the amount of water secreted and the osmotic pressure of the urine. Vasopressin mediates its effect by producing an increase in water permeability in its target renal cells (van Lieburg et al. 1995). On one side it helps to build up the osmotic gradient in the loop of henle and on the other side it controls the water reabsorption by the collecting duct. Both effects together help to control the osmotic pressure of the urine and the amount of water secreted, regulating thereby the osmotic pressure of the plasma.

Calcitonin is secreted by the parafollicular cells of the thyroid in response to elevations in serum calcium. The main function of calcitonin is the decrease of high calcium and phosphate concentrations in the blood by stimulation of bone formation by osteoblasts. In the kidney, calcitonin decreases the renal tubular reabsorption of calcium and phosphate as well as that of sodium, potassium and magnesium. Calcitonin affects the activity of the Na+/H+-exchanger and the Na+/K+-ATPase in a cell-cycle-dependent mechanism (Chakraborty et al. 1994). In this way calcitonin contributes to a reduction of the calcium and phosphate concentrations in the blood. Calcitonin, like VP, binds to specific renal receptors which activate adenylate cyclase (Lin et al. 1991).

3.6.2 cAMP-Dependent uPA Gene Expression in LLC-PK1 Cells

Due to the presence of the vasopressin and calcitonin receptors, the LLC-PK1 cells provide an attractive system for studying the cAMP-dependent pathway. LLC-PK1 cells possess both the type I and II PKA isotypes in about equal proportions (Hemmings, 1985). uPA gene expression is strongly induced upon stimulation of the cells with
agents that raise intracellular cAMP concentration such as calcitonin, the \( \alpha \)-subunits of the G-protein, ADP-ribosylating cholera toxin, the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) and cAMP itself (Dayer et al. 1981). Mutant cell lines, which are affected in components of the cAMP-pathway upstream of PKA, are impaired in uPA gene regulation by cAMP (Jans et al. 1987).

The cAMP-dependent uPA gene expression is a transcriptional event as shown in vivo (Nagamine et al. 1983) under cell-free conditions. Transcription of uPA templates in a cell extracts of LLC-PK\(_1\) cells can be stimulated by the addition of either cAMP or the purified catalytic subunit of protein kinase A (Nakagawa et al. 1988). Nuclear run-on transcription experiments indicated that uPA transcription was strongly induced by the treatment of cells with calcitonin and 8-bromo-cAMP (Degen et al. 1985; Andrus et al. 1988). Calcitonin induction of uPA mRNA accumulation was not suppressed by a 30 minutes pretreatment of the cultures with as much as 100\( \mu \)g/ml cycloheximide and cycloheximide by itself did not increase uPA mRNA concentrations (Nagamine et al. 1983).

The effect of cAMP on the uPA gene in LLC-PK\(_1\) cells is a phenomenon which is not found in other cells and therefore is a appealing example of cell-specific gene regulation (see 3.6.2.2).

Taken together these results indicate that calcitonin induces uPA-gene expression utilizing factors already present before the administration of the hormonal stimulus. From these results, the most interesting question was, what are the \textit{cis}-acting elements mediating cAMP action in the uPA promoter?

3.6.2.1 The ABC-Enhancer

The first search for sequences which could lead to cAMP-dependent uPA gene expression was undertaken in a cell-free transcription analysis study with a construct that contained 1.3 kb of the uPA 5'-flanking region (Nakagawa et al. 1988). A template with only 148 bp of 5'-flanking region was as responsive to cAMP induction as the 1.3 kb construct. In this 148 bp fragment three potential sites were detected: two of them are SP1 consensus sites and a GAGTCA-site which is similar to half of the AP1-binding site TGA(G/C)TCA. One limitation of the cell-free transcription system is the lack of histones on the DNA and that the DNA is not integrated into the chromosome as the template was exogenously provided as naked DNA. Furthermore under cell-free conditions proteins may have lost activity during the preparation of the extracts or the concentrations of the factors may have been altered.
In another approach, to test uPA gene regulation under more physiological conditions, hybrid genes composed of different lengths of the uPA promoter were linked to a reporter gene (CAT) and stably transfected into LLC-PK1 cells (von der Ahe et al. 1990). The longest construct comprised 4660 bp of the pig uPA promoter. Stably transfected cells were treated with Br-cAMP or salmon calcitonin for two hours and the amount of specific RNA transcripts was quantified. When the region between -3473 and -2693 upstream of transcription initiation site was deleted the most prominent reduction in cAMP-inducibility was observed.

Within the area between -3473 and -2693, three CRE-like sites were detected: -3407\textsuperscript{TGACGcac-3400}, -3389\textsuperscript{TGACGaac-3382} and -2842\textsuperscript{TGACGctC-2849}. The two first were studied in more detail and are the subject of the present work. There are most likely multiple cAMP-responsive sites scattered throughout the whole uPA-promoter (von der Ahe et al. 1990). It was also shown that the strong cAMP induction of the uPA gene requires alterations of the chromatin structure which allow the cooperative interaction between the multiple cAMP-responsive sites (Lee et al. 1994a).

The area between an Xhol-site at -3473 and an Nsil-site at -3324 of the uPA-gene promoter was alone able to mediate cAMP-responsibility but was unable to mediate TPA-induction when placed in front of a heterologous promoter (von der Ahe et al. 1990). Therefore this 149 bp fragment contained elements which are sufficient to mediate cAMP-responsiveness. In DNaseI footprinting analysis experiments of this region five protein binding sites were detected termed FP-A, -B, -C, -D and -E. Only FP-A, -B and -C were apparently really involved in the cAMP-responsiveness. The areas of these footprints were further studied in methylation interference experiments (von der Ahe et al. 1990). The results of these studies are shown in Figure 8.

The cAMP-responsive area was narrowed down to the sequence between -3342 and -3414. Both footprints A and B contain a TGACG-element in their centers. This motif is the half-palindromic part of the cAMP-responsive element (CRE), which is described in 3.3.3. FPC is different from FPA and FPB in that it does not contain a CRE-like site and is much longer than the other two binding sites.
Figure 8: Sequence around a cAMP responsive site 3.4 kb upstream of the transcription initiation site. Promoter proximal cis-acting elements are described in (von der Ahe et al. 1988). Footprints are labeled as A, B, C, D and E. The sequence of the enhancer that contains XhoI-NsiI fragment is shown on the bottom. Numbers refer to the distance from the initiation of transcription. Brackets indicate DNA sequences protected from DNase I by nuclear factors. Regions of weak protection are indicated by stars. The positions of apparent protein contacts, as determined by the methylation interference assay, are indicated by closed triangles. The figure is kindly provided by (von der Ahe et al. 1990).

Figure 9: The DNA binding sites in the ABC-enhancer differ strongly from consensus binding sites. The sequence of the pig uPA promoter between -3342 and -3414 is shown. Shown below are the consensus binding sites for ATF/CREB and for LFB3.

Later, the protein which binds to FPC was purified by affinity column chromatography. Its partial sequencing identified it as LFB3/vHNF1 (Menoud et al. 1993). The LFB3 binding site differs remarkably from the consensus LFB3 DNA binding site. Most likely two LFB3-binding sites are present in FPC. An alignment of the consensus CREs and consensus LFB3-binding sites is shown Figure 9. The methylation interference assay showed that the protein binding to the FPA bound more strongly to the sequence upstream of the CRE-like site, whereas the protein binding to
the FPB rather interacted with the sequence downstream of the CRE-like site. The DNA sequences protected from DNaseI digestion furthermore support the above idea, that the protein at FPA binds more upstream of the CRE-like site than the protein at FPB does. The conclusions from these considerations are that the proteins binding to FPA and FPB may be distinct. FPA, -B and -C are only weakly inducible by cAMP, but multimerization of FPA or FPB leads to cAMP-inducibility. Most interestingly the orientation of FPA and FPB is important, since FPA or FPB in isolation show better induction by cAMP if they are inverted (von der Ahe et al. 1990). The binding activity of nuclear proteins to FPA and FPB is increased by the addition of the catalytic subunit of the cAMP-dependent protein kinase, whereas the binding to FPC is not affected by this treatment as determined by gel retardation studies (von der Ahe et al. 1990). This result fits with the fact that LFB3 does not contain any putative PKA-phosphorylation site. Competition analysis of the three protein binding domains revealed that oligonucleotide complexes with the FPA- and FPB-sequences detected in gel retardation experiments can be competed with oligonucleotides containing the somatostatin CRE- and human chorionic gonadotropin α-subunit-sequences. The most interesting observation was that oligonucleotides A and B were competed not only by themselves but also by an oligonucleotide containing the FPC-sequence. This result indicates a protein-protein interaction between the proteins binding to FPA and -B and LFB3-protein (von der Ahe et al. 1990). The ABC-enhancer seems to serve as an interesting model where tissue-specific gene expression is coupled with hormonal regulation. A model was therefore proposed where CREB or CREB-like proteins bind to the FPA and FPB upon being phosphorylated after hormonal stimulation.

3.6.2.2 Other Potential cAMP-Response Elements in uPA-Promoters of Various Species

Another site in the pig uPA promoter which can mediate cAMP-responsiveness was found to be located at -2530 and -2350 upstream of transcription initiation site (Lee et al. 1994a). This 180 bp-area also contains a CRE-like element. This region is conserved between the pig and the mouse uPA gene promoters (Cassady et al. 1991) and was shown to confer cAMP-inducibility on a heterologous promoter (Lee et al. 1994a). This site was not the object of further research.

The region of the ABC-enhancer has no homology with the murine uPA promoter, implying that it evolved only very late. Unfortunately the sequence of the corresponding region in the human promoter is not yet available. Therefore it is not clear if this area is also present in the human promoter. Despite the absence of the ABC-enhancer in the
murine uPA-promoter, the uPA gene is inducible by cAMP in different mouse cell lines, indicating that other enhancers must exist which mediate cAMP-responsiveness in mouse. Other cAMP-responsive sites were also found in the pig uPA gene promoter, of which one close to the transcription start site is described above.

Induction of uPA after an increase in intracellular cAMP-concentration occurs in many different cell lines from different species. For most of these cases only the fact that they produce uPA was shown but no further investigation of the site which mediates cAMP-induction was undertaken. In primary mouse Sertoli cells, two sites mediate cAMP-induction of the uPA gene. One of these sites is located between -72 and -29 bp upstream of transcription initiation site, where a putative AP-2 site is located (Rossi et al. 1990). Further dissection of this area led to the identification of changes in DNA-protein complexes after treatment of the cells for 12 hrs with cAMP. This site was located between -54 and -42 and had the sequence \(^{53}\text{GGGAGGGGCG}^{43}\). Mutation of this site abolishes cAMP-responsiveness. Two proteins bind to this area with partially overlapping DNA-binding sites (Grimaldi et al. 1993). In mouse ovarian granulosa cells, uPA is produced after stimulation with Bt\(_2\)cAMP, with a concomitant increase in uPA mRNA level (Canipari et al. 1987). In the human epidermoid carcinoma cell line A431, EGF and TPA induce uPA production through alternative pathways. 8-Br-cAMP had no effect by itself, however it inhibited the effects of EGF and doubled the TPA induction (Kessler et al. 1991). Shionogi mouse mammary carcinoma cells (SC115) secreted uPA after stimulation with 8-Br-cAMP. The effect of cAMP is potentiated by addition of retinoic acid together with cAMP. This increase was also detectable by Western blot and Northern blot analysis and could be inhibited by actinomycin D (Mira-y-Lopez, 1991). In the human pre-B lymphoma cell line RC-K8, a cell-line constitutively secreting uPA, uPA gene activity was repressed by Bt\(_2\)cAMP. The PKA-inhibitor H89 strongly inhibited PKA activation and the reduction of uPA mRNA levels after cAMP addition. The repression by cAMP is also inhibited by the presence of cycloheximide (Shinbo et al. 1995).
4. MATERIALS AND METHODS

4.1 Reagents

12-O-tetradecanoylphorbol-13-acetate (TPA), gelatin for F9 cell-culture, colchicine and cytochalasin B were obtained from Sigma, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) from Fluka, 8-bromo-cAMP (Br-cAMP) from Boehringer Mannheim, and okadaic acid from Anawa. [α-32P]dATP (3000 Ci/mmole) and [α-32P]UTP (800 Ci/mmole) were obtained from Amersham. The oligonucleotides used for electromobility shift assays were (only the sense strands are given):

domain A, 5'-'AATTCTGTGCCTGACGCACAG-3'
domain B, 5'-AATTCCATGCAGCAACACTGGG-3'
domain C, 5'-GTGAATGATAAAGGAATTAAGATGATTCAC-3'
mPEA3/AP1, 5'-GATCCGTCAGAGAGGTAAGTACGCTCCTG-3'
SP1, 5'-GATCCAGCCCTGCGCCGCTAGCCTG-3'
somatostatin CRE, 5'-AATTCGCCCTCCTGAGCTCAGAGAGAGAG-3'

The mPEA3/AP1 sequence was based on the PEA3/AP1 site of the uPA gene but its PEA3 site has been mutated (Menoud et al. 1993). The somatostatin CRE sequence was derived from the rat promoter and comprises the sequence between the NarI and DraI restriction sites located at -382 and -413, respectively (Montminy et al. 1986). The sequences of the oligonucleotides used in the construction of templates are shown in Figure 10.

Figure 10: Constructs with mutations in domains A, B or C. Mutated sequences are indicated in small letters. The domains A, B and C are labeled at the top of the figure.
4.2 Cell Culture

LLC-PK₁ (Hull et al. 1976), 293 and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum (AMIMED), 0.2 mg/ml streptomycin, and 50 units/ml penicillin, at 37°C in a humidified CO₂ (5%) incubator. NIH 3T3, F9 and HeLa cells were cultured in the above Dulbecco's medium supplemented with 5% calf serum for NIH 3T3, 5% fetal calf serum for F9 cells and 3% fetal calf serum and 3% newborn calf serum for HeLa cells. All cell lines were plated directly onto plastic dishes except for the F9 cells which were plated onto gelatin-coated plastic dishes.

4.3 Expression Vectors

In pTATA (kindly provided by A. E. Sippel) the firefly luciferase gene is linked to a minimal promoter of the thymidine kinase gene (-46 to +52) containing only the TATA box and the transcription initiation site. Mutated and non-mutated sequences derived from the cAMP-responsive enhancer ABC site containing AccI-BglII ends, were inserted immediately 5' of the TATA box of pTATA into the AccI-BamHI sites at positions -209 and -196. The sequences used are shown in Figure 10. In p3API-TATA, three consensus collagenase API elements were inserted into the same AccI-BamHI sites of pTATA. The sequence of the oligonucleotide of the three API elements was: 5'-cgacGGGCTGACTCATCACGGCTGACTCATCAa-3'. In pCRE-TATA the rat somatostatin CRE-sequence was used: 5'-cgacGGCCTCCTTGGCTGACGTCAGAGAGAGTTTa-3'. Similar constructs with non-mutated sequences, but with the SV40 early gene promoter and the pig LFB3 expression vector (RSV-FPCB) have been described previously (Menoud et al. 1993). Mutations of the pig LFB3 expression vector, referred to as B, C, D, E and F, were made by inserting EcoRI-BamHI fragments, encoding the regions of 150-245 bp, 245-1829 bp, 150-720 bp, 150-1082 bp or 150-1448 bp of pig LFB3, by PCR into the pSG5 expression vector (Green et al. 1988). The pAlb-luc construct was made by subcloning the 190 bp HindIII-BglII fragment of the rat albumin proximal promoter from pAlb-cat (kindly provided by M. Yaniv, described in (Heard et al. 1987)) into the HindIII-BglII sites of pGL2-basic (Promega). The CBP-expression vector pCMV-CBP was provided by B. Lüscher (Oelgeschlaeger et al. 1996). The expression vector for the catalytic subunit of PKA (pCEV) was a generous gift from S. McKnight. The pJ6-ATF1 expression vector was provided by P. Verde. The 12S Ela expression vector was provided by J.R. Nevins (Weigel et al. 1990).
4.4 Plasmids and Probes

The cDNA clones for pig uPA, pYN15 (Nagamine et al. 1984), for pig actin, pACT4 (Ziegler et al. 1991), and for pig LFB3 (Menoud et al. 1993) have been described. The DNA insert from each plasmid was labeled with \([\alpha^{32}P]dATP\) by random priming (Feinberg et al. 1983).

4.5 Transient Transfection Assays

3 x 10^5 (LLC-PKI) or 2 x 10^5 (F9) cells per 35-mm plate were seeded the day before transfection. The cells were transfected using calcium phosphate-mediated precipitation with 1–3 \(\mu\)g of DNA. The DNA was dissolved in 40 \(\mu\)l distilled water and mixed with 40 \(\mu\)l buffer A (0.5 M CaCl\(_2\) in 0.1 M HEPES/NaOH buffer pH 6.95) and incubated for 5 minutes. 80 \(\mu\)l buffer B (0.28 M NaCl, 0.75 mM NaH\(_2\)PO\(_4\), 0.75 mM Na\(_2\)HPO\(_4\) in 0.05 M HEPES buffer pH 6.95) was added, vortexed and incubated for 15 minutes. The calcium phosphate DNA precipitate was added to the cell culture without the removal of the medium. After 4.5 hours of incubation with LLC-PKI cells, the medium was removed and the cells were incubated for 1 minute in PBS- with 10\% DMSO, washed twice in PBS+ and incubated with normal medium under the conditions mentioned in 4.2. For F9 cells the calcium phosphate DNA precipitates were left on the cell cultures, without DMSO treatment, until the cells were harvested. When cells were to be induced, they were treated, 20 h after transfection, with 1 mM Br-cAMP for 6 h. The cells were harvested in 200 \(\mu\)l luciferase lysis buffer (25 mM glycylglycine-NaOH, pH 7.8, 1 mM DTT, 15\% glycerol, 8 mM MgSO\(_4\), 1 mM EDTA and 1\% Triton X-100). 20 \(\mu\)l of the samples were transferred to microtiter plates (Microlite 1; Dynatech) and luciferase activity was measured in a luminometer (Autolumat LB 953, Berthold) after the addition of 100 \(\mu\)l of luciferin solution (25 mM glycylglycine-NaOH, pH 7.8, 10 mM MgSO\(_4\), 1.5 mM ATP, 330 \(\mu\)M luciferin [Chemie Brunschwig AG]).

4.6 RNA Isolation and Northern Blot Analysis

Total RNA was isolated according to Chomczynski and Sacchi (1987), and analyzed for levels of specific mRNAs by Northern blot hybridization as described (Ziegler et al. 1991). The cells plated on 35 mm dishes were harvested, after having been washed twice with PBS-, in 0.4 ml solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5\% sarcosyl, 0.1\% \(\beta\)-mercaptoethanol). 40 \(\mu\)l 2 M sodium acetate pH 4.0, 0.4 ml water saturated phenol and 80 \(\mu\)l chloroform-isoamylalcohol (49:1) were added, vortexed and the supernatant was precipitated with 0.45 ml isopropanol. The precipitate was washed with cold ethanol and dissolved in solution D and reprecipitated with isopropanol. Finally, the precipitate was dissolved in water and the RNA
concentration was measured by determining the absorbance at 260 nm. Total RNA (5 µg/lane) was fractionated on a 1.1% agarose gel containing 3% formaldehyde, 0.1% SDS, 2 mM EDTA (pH 8.0) and 40 mM triethanolamin at 65V and blotted onto a nylon membrane (Boehringer) as described (Nagamine et al. 1983). RNA was fixed by irradiating the membrane with UV. To confirm the loading and transfer of similar amounts of RNA, ribosomal RNA was visualized on nylon filters by staining with 0.04% (w/v) methylene blue in 500 mM sodium acetate pH 5.2 and subsequent destaining with 40% ethanol (Herrin et al. 1988). Membranes were prehybridised with hybridisation buffer (50% formamide, 0.2 mg/ml single stranded heringsperm DNA, 10 mM EDTA pH 8.0, 10 mM pipes pH 6.4, 10 x Denhardt’s solution, 5 x SSC and 1% [w/v] SDS ) for 6 hours at 42°C. Hybridisation was performed overnight at 42°C with hybridisation buffer containing 2 x 10^6 cpm of radioactive probe. Prior to the addition to the hybridisation buffer, the probe was treated by heating at 56°C for 2 minutes in 50% formamide solution. The filters were washed three times with 1% SDS and 1 x SSC at 55°C and exposed to Kodak X-Omat AR film with an intensifying screen at -70°C. Levels of specific RNA were quantified using a Molecular Dynamics PhosphorImager.

4.7 Determination of mRNA Stability

RNA stability was measured by the RNA synthesis inhibitor-chase method as described (Ziegler et al. 1990). Cells were treated with DRB (20 mg/ml) to inhibit transcription and total RNA was isolated at several subsequent time points. The RNA was analyzed by Northern blot hybridization. mRNA levels were plotted in a half-logarithmic scale using SigmaPlot (Jandel Scientific) and subjected to linear regression.

4.8 Nuclear Transcription

The isolation of nuclei, nuclear run-on transcription, and quantification of specific transcripts by hybridization were performed as previously described (Andrus et al. 1988). Filters were prepared by linearization of 1-2 µg of the plasmids with an appropriate restriction enzyme. Subsequently, the DNA was denatured in 230 µl total volume of 20 mM NaCl and 0.5 M NaOH at 80°C. On ice, the pH of the solution was subsequently neutralized with the same amount of 1 M HCl and buffered with 25 µl of 1 M Tris-HCl pH 7.6 and 230 µl 20 x SSC. The solution was applied on a slot blot apparatus with a nitrocellulose membrane (Schleicher & Schuell). The membrane was rinsed with 10 x SSC and baked for 2 hours at 80°C under vacuum. The cell extracts were prepared on ice. LLC-PK1 cells (3 x 10^7 cells/150 mm dish) were rinsed twice with 25 ml PBS-, twice with 25 ml buffer 1 (0.3 M sucrose, 1.5 mM MgCl2 and 10 mM Tris-HCl pH 7.9). Then 25 ml buffer 2 was added (10 mM KCl, 1 mM MgCl2 and 10 mM
Tris-HCl pH 7.9) and incubated for 10 minutes. Buffer 2 was removed and 5 ml buffer 3 (0.3 M sucrose, 1.5 mM MgCl₂, 0.3% (v/v) Triton X-100 and 10 mM Tris-HCl pH 7.9) was added and cells were collected with a rubber policeman. The cells were homogenized by 15 strokes with a Teflon homogenizer and 10 ml of buffer 4 (40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 50 mM Tris-HCl pH 8.3) was added. The nuclei were twice sedimented by centrifugation at 700 × g for 10 minutes with an intermediate wash of the cells with 10 ml buffer 4. Finally the cells were suspended in 200 μl buffer 4. 100 μl of this suspension was used for radioactive transcription in the presence of 10 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 0.3 M KCl, 0.5 mM CTP, 0.5 mM ATP, 0.5 mM GTP and 100 μCi [α-³²P]UTP, 2.5 mM DTT and 40 units RNase Inhibitor (Boehringer) at 30°C for 30 minutes. Then, 40 μM UTP was added for 2 minutes. The reaction was stopped with 400 μl solution D, followed by phenol extraction with the solutions described under 4.6 using 60 μl of 2 M sodium acetate, 600 μl water saturated phenol and 120 μl chloroform-isoamylalcohol. The solution was centrifuged at 8,000 rpm for 10 minutes and the supernatant was precipitated by 650 μl isopropanol and 1 μl E. coli tRNA (10 mg/ml). The pellet was washed with 75% ethanol dissolved in 300 μl solution D and precipitated again with 300 μl isopropanol. The precipitated RNA was dissolved in 100 μl TE and applied on a sephadex G50 column. The eluate was precipitated as described above and finally dissolved in TE. Equal amounts of radioactivity was used for hybridisation using a slightly modified hybridisation buffer (200 μg/ml tRNA, 10 mM EDTA pH 8.0, 10 mM Pipes pH 6.4, 5 × SSC, 5 × Denhardt’s solution and 0.1% SDS). Hybridisation was performed at 45°C for 2 days. Filters were washed twice with 5 × SSC, 0.2% SDS, twice with 2 × SSC, 0.2% SDS and for 30 minutes at 37°C with 2 × SSC and 10 μg/ml RNase A. Filters were dried and exposed as described in 4.6.

4.9 Nuclear Extracts and Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from LLC-PK₁ cells and electrophoretic mobility shift assays were performed as previously described (Lee et al. 1993). Cells were plated onto 150 mm dishes and washed with 25 ml PBS- scraped with a rubber policeman and precipitated with centrifugation at 1000 rpm at 4°C for 5 minutes. The pellet was lysed in 400 μl lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μg/ml leupeptin) by 15 strokes in a Teflon homogenizer. The lysed cells were centrifuged at 2,000 rpm at 4°C for 5 minutes. The pellet was rinsed with PBS- and resuspended in 100μl extraction buffer (20 mM Tris-

HCl pH 8.0, 0.5 mM EDTA, 500 mM NaCl, 25% glycerin, 1 mM PMSF and 2μg/ml
leupeptin). The suspension was incubated for 15 minutes at 4°C and centrifuged for 15 minutes. The supernatant was aliquoted and stored at -80°C. DNA-binding activity was analyzed in an electromobility shift assay using a gel with 1 × TAE, 5% acrylamide:N,N'-methylenebisacrylamide (29:1), 0.1% ammoniumpersulfate and 1 µl TEMED. 1 µg/lane of these protein extracts, in a volume of 5 µl adjusted with extraction buffer, were incubated on ice for 15 minutes with 15 µl binding buffer (20 mM HEPES pH 7.9, 5 mM KCl, 0.2 mM EDTA, 2% Ficoll 400 [Pharmacia], 50 µg/ml Poly(dI-dC) [Pharmacia], 5 ng/ml single stranded Hering sperm DNA, 1 mg/ml BSA and 20 fmol radioactive DNA-probe). The gel was run at 130 V, dried and exposed as outlined above.

Radioactive probes were generated by annealing the oligonucleotides of the upper and lower strands and labeling them using the Klenow fill-in reaction with [α-32P]dATP. Supershift experiments using anti-ATF1 (Santa Cruz, Fl-1) and anti-CREB antibodies (Santa Cruz, X-12) or anti-GST-antibodies (Santa Cruz, Z.5) as non-specific antiserum were performed as described in (De Cesare et al. 1995). Nuclear extracts were incubated for 4 hours at 4°C with 2 µl of the respective antiserum prior to the addition of the radioactive probe. Thereafter samples were analyzed on 0.25 × TBE-gels containing 5% acrylamide:N,N'-methylenebisacrylamide (29:1), 0.1% ammoniumpersulfate and 1 µl TEMED at 200 V for 2 hours and exposed to Kodak X-Omat AR film with an intensifying screen at -70°C.

4.10 Protein Kinase A Treatment of Nuclear Extracts

Nuclear extracts were prepared as described in 4.9 and treated with protein kinase A according to (Dai et al. 1996). 10 µl of nuclear extracts, containing 16 µg of protein, were incubated with 4 µl of 10 × kinase buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 12 mM MgCl2 and 2.5 mM ATP) and 4 µl (100 units) catalytic subunit of protein kinase A (Calbiochem) for 90 minutes at 37°C. Afterwards, samples were incubated with 2 µg of antibodies for four hours at 4°C. The samples were assayed for DNA-binding activity as described in 4.9.
5. RESULTS

5.1 Role of LFB3 in Cell-Specific cAMP Induction of the Urokinase-Type Plasminogen Activator Gene


5.1.1 Summary

In previous work we suggested that a kidney-specific transcription factor LFB3 cooperates with cAMP-response element (CRE)-binding proteins within a cAMP regulatory unit comprised of three protein-binding domains and located 3.4 kb upstream of the urokinase-type plasminogen activator (uPA) gene in LLC-PK₁ cells (Menoud et al. 1993). The two domains contain a CRE-like sequence and the third domain is recognized by LFB3. The absolute requirement of LFB3 as well as the cooperation among the three domains for cAMP-regulation were confirmed by transient transfection assays in F9 teratocarcinoma cells, in which the level of LFB3 was negligible. Suspecting a possible feedback regulation of LFB3 mRNA expression during cAMP-dependent uPA gene induction in LLC-PK₁ cells, we measured LFB3 mRNA levels after cAMP treatment and found a strong reduction. This reduction was not due to a change in template activity of the LFB3 gene because run-on transcription showed no significant change in LFB3 gene transcription. RNA synthesis inhibitor-chase experiments indicated that the down regulation was post-transcriptional. Interestingly, when the inhibitor was added at the same time as cAMP, the cAMP-induced decrease in LFB3 mRNA levels was abrogated, suggesting that on-going RNA synthesis is required for the decrease. Similar effects on LFB3 mRNA metabolism were observed with all agents that induce uPA mRNA in LLC-PK₁ cells, including 12-Î”-tetradecanoylphorbol-13-acetate, okadaic acid, colchicine and cytochalasin. We discuss the significance of this regulation in uPA gene expression.

5.1.2 Introduction

Signal transduction, a process of successive activation of various molecules, is subject to various levels of regulation. In many cases, for the sake of homeostasis, activated molecules are sequestered from the pathway by desensitization of membrane-bound receptors (Soderquist et al. 1986; Sibley et al. 1987), degradation of activated molecules (Hemmings, 1986; Young et al. 1987), inactivation of activated molecules by dephosphorylation (Hagiwara et al. 1992; Depaoli Roach et al. 1994), or by a feedback mechanism (Beullens et al. 1993). Cross-talk between different signaling pathways is also an important mechanism for bestowing flexible and versatile regulation on a given pathway. This can be either positive or negative and occurs at various steps in the
pathway in a cell-specific manner (see reviews Darnell, Jr. et al. 1994; Delmas et al. 1994; Hunter, 1995). Therefore, in addition to the identification of successively activated components of a signaling pathway and the elucidation of the mechanism of activation of each component, it is also very important to know how the activity of each component is modulated by molecules not immediately upstream in the pathway. In this way, the nature of a signaling pathway may be understood in a more physiologically relevant context.

We have been studying urokinase-type plasminogen activator (uPA) gene regulation in LLC-PK₁ cells, a cell line derived from pig kidney epithelia (Hull et al. 1976). In these cells, the uPA gene is induced through independent signaling pathways by various signals such as cAMP (von der Ahe et al. 1990), 12-O-tetradecanoylphorbol-13-acetate (TPA) (Lee et al. 1993), the protein phosphatase 1/2A inhibitor okadaic acid (Nagamine et al. 1991b; Lee et al. 1994b) and cytoskeletal reorganization (Botteri et al. 1990; Lee et al. 1993). The pig uPA gene has a cAMP-inducible enhancer located 3.4 kb upstream of the transcription start site (von der Ahe et al. 1990). This enhancer is comprised of three protein-binding domains, A, B and C. Domains A and B contain a core sequence of the cAMP response element (CRE) but require the adjoining C domain to confer full cAMP responsiveness on a heterologous promoter (von der Ahe et al. 1990; Menoud et al. 1993). The C domain has no CRE and cannot mediate cAMP responsiveness when used in isolation. We have purified the protein binding to the C domain (Menoud et al. 1993) and found it to be the pig equivalent of mouse LFB3 (De Simone et al. 1991). It is also known as HNF1β (Mendel et al. 1991b) or vHNF1 (Rey-Campos et al. 1991). LFB3 is a tissue-specific transcription factor highly expressed in kidney cells (De Simone et al. 1991) with a structure closely related to the liver-specific transcription factor HNF1α. Both HNF1α and LFB3 recognize the same DNA sequence, at least in vitro (Bach et al. 1991; Menoud et al. 1993), although the domain C sequence is quite different from the consensus HNF1α recognition sequence. It is still not known which genes besides the uPA gene are the targets of LFB3 in kidney cells, or how the expression of LFB3 is regulated. As LFB3 is apparently involved in cAMP-dependent uPA gene regulation in LLC-PK₁ cells, we were interested to know whether cAMP-evoked signaling affected the expression of LFB3 in these cells. Indeed, we have shown that cAMP treatment strongly reduces LFB3 mRNA levels, suggesting a feedback mechanism via LFB3 in cAMP-dependent uPA gene regulation in LLC-PK₁ cells (Menoud et al. 1993). In the present study, we verify the involvement of LFB3 in cAMP-induction of the uPA gene and show that not only cAMP but also other agents that induce uPA gene expression strongly reduce the amount of LFB3 mRNA. These agents are 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, colchicine, and
cytochalasin B. Our results suggest the involvement of LFB3 in uPA gene regulation by cAMP at different levels.

5.1.3 Results

5.1.3.1 Cooperation of LFB3 in a cAMP-Responsive Enhancer

A cAMP-inducible enhancer located 3.4 kb upstream of the transcription initiation site of the uPA gene is composed of three protein binding domains, A, B and C; domain C lacks a CRE sequence and is necessary together with domains A and B for full cAMP-inducible activity (von der Ahe et al. 1990). Cooperation between domains A and B and domain C was re-evaluated in the context of the minimum thymidine kinase gene promoter containing only a TATA box by transient transfection assays (Figure 11b). To activate cAMP-dependent signaling, we used Br-cAMP or an expression vector of the catalytic subunit of cAMP-dependent protein kinase. As shown in Figure 11b, templates with domains A and B alone or domain C alone did not exhibit significant inducibility compared with the control template, while the template with all three domains (pABC-TATA) showed strong inducibility. This inducibility was strongly reduced when the template pABC-TATA was mutated in any of the three domains. The mutations were introduced to the sites that had been shown to interact with nuclear proteins by methylation interference experiments (von der Ahe et al. 1990). The induction with Br-cAMP elicited a stronger response than with the catalytic subunit, which may be due to a high concentration of free regulatory subunits in LLC-PK1 cells.

We previously cloned the domain C-binding protein and found it to be the pig equivalent of mouse LFB3 (Menoud et al. 1993). We therefore examined the effect of LFB3 on the above templates by transient coexpression assays in F9 cells, which have a negligible level of endogenous LFB3. We used only the catalytic subunit to activate the signaling because endogenous cAMP-dependent protein kinase is not responsive to cAMP in F9 cells by an unknown mechanism (Masson et al. 1992). Figure 11c shows that in F9 cells pABC-TATA was strongly induced by the catalytic subunit only when LFB3 was coexpressed. The control pTATA was not affected. These results unambiguously indicate the cooperation among three protein-binding domains and the involvement of LFB3 in cAMP regulation through the ABC site.
Figure 11a: Cooperative role of domain C with neighboring domains A and B in uPA gene induction by cAMP signaling. Luciferase gene constructs containing different parts of a cAMP-inducible enhancer of the uPA gene which is composed of domains A, B and C. The positions of apparent protein contacts as determined by methylation interference experiments are indicated by stars. Mutated domains and sequences are indicated by small letters.

5.1.3.2 Effect of cAMP and other uPA Inducers on LFB3 mRNA Levels

To confirm the previous observation that cAMP treatment reduces LFB3 mRNA in LLC-PK$_1$ cells, we compared Br-cAMP to other agents shown to induce uPA mRNA in the same cells: TPA, colchicine, cytochalasin B and okadaic acid. The cells were incubated for the time optimal for uPA mRNA induction, i.e., 2 h for Br-cAMP and TPA and 4 h for the rest. As shown in Figure 12, Br-cAMP as well as other agents strongly reduced LFB3 mRNA levels; all of them induced uPA mRNA. The greatest reduction in LFB3 mRNA levels was obtained with TPA and okadaic acid (85% by 2 h and 4 h) and the least with cytochalasin B (60% by 4 h).

With the exception of Br-cAMP, all the other agents induce uPA gene via the activation of AP1, acting on the PEA3/AP1 site located 2 kb upstream of the transcription initiation site (Lee et al. 1993b; Lee et al. 1994b). Therefore, in the following experiments we compared in particular Br-cAMP and TPA.
5.1.3.3 Effect of Br-cAMP and TPA on Domain C-Binding Activity

We tested whether the reduction of LFB3 mRNA levels after treatment with uPA inducers was reflected at the protein level. As specific antibodies against pig LFB3 were not available, we measured domain C-binding activity in nuclear extracts. We performed electrophoretic mobility shift assays using crude nuclear extracts prepared from LLC-PK₁ cells pretreated for 7 h with Br-cAMP or TPA. Using a ³²P-labeled domain C oligonucleotide as a probe, we observed a single distinct band (Figure 13a), which could be competed by excess of the identical unlabeled oligonucleotide but not by an oligonucleotide carrying the same mutations as shown in Figure 11a (Figure 13b). After treatment of the cells with TPA, the binding activity was reduced to about 50%,
with Br-cAMP to 40% and with TPA and Br-cAMP added together to about 30%. These data indicate that the LFB3 binding activity is reduced by Br-cAMP or TPA treatment, suggesting that the reduced mRNA level affects the protein level. To test if the observed decrease in domain C-binding activity after 7 hrs TPA treatment is only a transient effect, we prepared nuclear extracts of LLC-PK1 cells treated for 12 and 24 hrs and measured their binding activity to the C-domain. As shown in Figure 13c, the binding activity was markedly decreased also after 24 hrs, suggesting that the effect is sustained over 24 hrs. To see whether the observed reduction is specific for domain C binding, we tested other oligonucleotides recognized by different transcription factors using the same nuclear extracts. Although domains A and B were required for cAMP induction, proteins binding to these sites were not affected by treatment of the cells with Br-cAMP or TPA (Figure 13a). With the SP1 oligonucleotide, two major specific bands were detected, but they did not change in intensity after this treatment. In contrast, with the mPEA3/AP1 oligonucleotide, which binds transcription factor AP1 (Lee et al. 1993), the intensity of the shifted band markedly increased on treatment with TPA and even more with Br-cAMP.

Figure 12: LFB3 mRNA levels. Total RNA was prepared from cells pretreated with 1 mM Br-cAMP or 100 ng/ml TPA for 2 h or 0.5 μM colchicine, 10 μM cytochalasin B or 125 nM okadaic acid for 4 h. Samples (5 μg each) were analyzed for the levels of LFB3 and uPA mRNAs by Northern blot hybridization.
Figure 13: Domain C-binding activity. a. Domain C-binding activity in the nucleus was tested by electromobility shift assays using crude nuclear extracts from LLC-PK₁ cells pretreated for 7 h with 1 mM Br-cAMP, 100 ng/ml TPA or both. As controls, the same extracts were tested for domains A and B, mPEA3/AP1 and SP1 binding activities. b. Binding specificity of nuclear extracts prepared from LLC-PK₁ cells to domain A, B and C. The complexes were either competed with the non-mutated respective oligonucleotide indicated by capitals or with the mutated sequence indicated by small letters. c. Effects of longer TPA-pretreatment on domain C-binding activity. Nuclear extracts prepared of LLC-PK₁ cells pretreated for 7, 12 and 24 hrs with TPA were assayed in electromobility shift experiments.
5.1.3.4 No Change in Transcription Rate of the LFB3 Gene

The mechanism leading to the reduction of LFB3 mRNA by uPA inducers may involve either transcriptional or post-transcriptional regulation of the LFB3 gene. To distinguish between these two possibilities, we first performed nuclear run-on transcription to assess changes in the LFB3 gene transcription rate. The results shown in Figure 14 indicate that the transcription rate of the LFB3 gene did not change when cells were treated with TPA, Br-cAMP, colchicine or cytochalasin B. As expected, these agents significantly enhanced the uPA gene transcription rate. Thus, the decrease in LFB3 mRNA levels seems not to be due to decreased de novo synthesis of LFB3 mRNA, suggesting that the reduction of LFB3 mRNA is a post-transcriptional event.

Figure 14: Nuclear run-on transcription. Nuclei were isolated from LLC-PK₁ cells untreated or pretreated with various agents for 90 minutes. Nuclear transcription was performed in the presence of a radioactive precursor and specific transcripts were analyzed by filter hybridization.
Figure 15: Stability of LFB3 mRNA. Effects of Br-cAMP (a and b) and TPA (c and d) on the stability of LFB3 mRNA were examined by RNA synthesis inhibitor chase experiments. In a and c, Br-cAMP and TPA, respectively, were added at the same time of DRB. In b and d, Br-cAMP and TPA, respectively, were added 1 h before DRB. ■, DRB; ○, TPA or Br-cAMP; □, DRB plus TPA or Br-cAMP.

5.1.3.5 Induced Instability of LFB3 mRNA

If a post-transcriptional step is responsible for the induced decrease in LFB3 mRNA level, the most obvious mechanism could be an effect on mRNA stability. The stability of LFB3 mRNA was assessed by DRB-chase experiments. Since DRB specifically inhibits the synthesis of eukaryotic heterogeneous nuclear RNA and mRNA (Tamm et al. 1978), chase of mRNA levels after its addition allows estimation of the decay rate of the mRNA. Because inhibition of mRNA synthesis may have some indirect influence on mRNA stability (Nanbu et al. 1994), we did chase experiments using two different schemes: in one experiment DRB was added at the same time as Br-cAMP or TPA, and
in the other DRB was added 1 h after Br-cAMP or TPA treatment. The effect on LFB3 mRNA was independent of the presence of Br-cAMP when DRB was added at the beginning of the experiment (Figure 15a). However, LFB3 mRNA decayed faster in the presence of Br-cAMP when DRB was added 1 h after Br-cAMP (Figure 15b). Similar results were obtained using TPA (Figure 15c,d). These results indicate that the stability of LFB3 mRNA is reduced by uPA inducers, and that this requires on-going RNA synthesis for at least 1 h at the beginning of the treatment.

5.1.3.6 Effect of the Decrease in DNA Binding Activity of LFB3 on cAMP-Induction

TPA and cAMP treatment reduced the DNA binding activity of LFB3. To test the biological relevance of this decrease in cAMP induction we asked whether TPA pretreatment could affect the cAMP-induction of pABC-TATA. As shown in Figure 16, TPA pretreatment by itself had little effect on basal expression but significantly reduced cAMP induction of the luciferase gene driven by ABC sites.

![Figure 16: Effect of TPA pretreatment on cAMP induction of pABC-TATA. LLC-PK1 cells were transfected with pABC-TATA. At 20 h after transfection cells were treated with or without TPA for 7 h, and then induced with or without 0.1 mM Br-cAMP for 4 h. Assays were done in duplicate and mean values are shown with error bars.](image-url)
LFB3 is a kidney-enriched transcription factor and plays a role as both positive and negative regulator of cAMP induction of the uPA gene through the ABC site. It allows cAMP induction by cooperating with CRE-binding proteins (CRE-BP) on the ABC site of the uPA gene promoter. But later on, it also mediates a negative feedback regulation by cAMP and TPA by decreasing its protein levels, which is due to enhanced degradation of LFB3 mRNA.

5.1.4 Discussion

LFB3 is an enhancer-binding protein augmenting basal expression of a gene that contains its cognate *cis*-element. We found in the induction of the uPA gene by cAMP in LLC-PK₁ cells that LFB3 is a positive regulator cooperating with CRE-binding proteins within a composite cAMP-responsive enhancer (Menoud et al. 1993; Marksitzer et al. 1995). Our results also suggest that LFB3 is involved in a down-regulating phase of cAMP-induced uPA gene expression. We have previously shown that uPA gene induction by cAMP is transient; the rate of uPA gene transcription reaches optimal after 2-4 h of cAMP treatment but declines thereafter (Degen et al. 1985). It may be that in uPA gene regulation LFB3 acts as a negative feedback regulator by decreasing its own concentration in response to cAMP. This throws new light on LFB3, which has been implicated as a factor coupling hormonal regulation and tissuespecific regulation of uPA gene expression in kidney epithelial cells (Menoud et al. 1993).

Decrease in domain C-binding activity seems to be due to a decrease in LFB3 protein levels. The decrease was also observed with TPA, and it may also be the case for colchicine, cytochalasin B and okadaic acid, which all decreased LFB3 mRNA levels (see below). These agents induce uPA gene expression in LLC-PK₁ cells via activation of the transcription factor AP1, although the mechanism of AP1 activation by each agent is different (Nagamine et al. 1991b; Lee et al. 1993b; Lee et al. 1994b). Thus, in addition to the features mentioned above, LFB3 may mediate negative cross-talk...
between cAMP-dependent signaling and AP1-activating signaling pathways in uPA gene regulation. Indeed, pretreatment with TPA significantly reduced cAMP induction of the luciferase gene driven by an enhancer consisting of domains A, B and C. The decrease in DNA binding by LFB3 in the cells seems to be due to the reduction in the protein levels. We cannot formally exclude the possibility that the decrease is due to a post-translational modification of the protein; however, this is in any case not the main cause because we also detected a strong reduction in LFB3 mRNA levels. The possible role of LFB3 in cAMP-dependent uPA gene regulation through the ABC-site revealed by this work is summarized in Figure 17.

The decrease in DNA-binding activity evoked by treatment with the uPA inducers in these cells was specific to the domain C binding protein, LFB3, and not a general effect, because DNA binding of the proteins recognizing domains A and B and of the ubiquitous transcription factor SP1 remained constant. Furthermore, the DNA-binding activity to the mutated PEA3/AP1-oligonucleotide, which contains an active API site mediating the action of TPA, colchicine, cytochalasin and okadaic acid, was increased by Br-cAMP as well as by TPA. We have not elaborated the mechanism of the increase in PEA3/AP1-binding activity, i.e., whether it is transcriptional or post-transcriptional. It is worthwhile to mention that the peptide hormone calcitonin, which raises intracellular cAMP concentrations, strongly enhances de novo synthesis of c-Fos and c-Jun (Lee et al. 1993), raising the interesting possibility of a cross-regulation of the TPA-dependent signaling pathway by the cAMP-dependent signaling pathway at the transcription step. The cAMP signal by itself does not utilize the PEA3/AP1 site to increase uPA gene expression (Lee et al. 1993). We do not know yet whether the enhancement of c-Fos together with c-Jun levels exerts positive effects on PEA3/AP1-site-mediated uPA gene expression, because the overexpression of c-Fos had no effect on uPA gene induction in NIH 3T3 cells (Besser et al. 1995).

The decrease in LFB3 mRNA levels is mainly attributable to induced mRNA instability. We did not detect changes in the LFB3 gene transcription rate but we did observe that LFB3 mRNA degradation increased in the presence of TPA or Br-cAMP. Interestingly, however, enhanced instability was observed only when DRB was added 1 h after TPA or Br-cAMP treatment, suggesting that some RNA transcripts or their translation products are involved in LFB3 mRNA metabolism. It may be that TPA or Br-cAMP induces a factor, RNA or protein essential for LFB3 mRNA degradation, or that an RNA or a protein of short half-life is involved in LFB3 mRNA degradation, at least at an early stage. A requirement for on-going RNA synthesis in mRNA degradation has been reported for several mRNAs, such as those for c-fos (Shyu et al. 1989), c-myc
(Feinberg et al. 1983), collagenase (Delany et al. 1992) and the transferrin receptor (Heckel et al. 1990). We have shown that an RNA instability-regulating site in the 3' UTR of uPA mRNA requires on-going RNA synthesis for its activity (Nanbu et al. 1994), and that the importance of this site in overall uPA mRNA degradation may depend on cell type (Stacey et al. 1994). In none of these cases, is it known how on-going RNA synthesis contributes to mRNA degradation.

Several instability-determining sequences have been identified in many mRNAs. These include sequences located in the 3'-untranslated region, such as the iron-responsive element in the transferrin receptor mRNA (Casey et al. 1989; Heckel et al. 1990), sequences in the unstable yeast MFA2 mRNA (Stacey et al. 1994) and AU-rich sequences in various oncogene and lymphokine mRNAs (Shaw et al. 1986). But instability-determining elements have also been identified in coding regions, e.g., c-myc (Feinberg et al. 1983) and c-fos (Shyu et al. 1991) mRNAs. We tested the 3' UTR and protein-coding regions of LFB3 mRNA in a system developed for the study of uPA mRNA degradation by inserting these sequences in an otherwise stable globin mRNA (Nanbu et al. 1994); however, the stability of recombinant globin mRNAs was not affected by TPA or Br-cAMP (unpublished data). It may be that regulatory sequences reside in 5' UTR or the 3' extreme which we have not tested or that the globin mRNA context interfered with TPA- and Br-cAMP-induced mRNA degradation.

Whether the cAMP and TPA signals utilize the same mechanism to induce LFB3 mRNA destabilization is not yet established, although it is plausible considering that induced LFB3 mRNA instability by either agent requires ongoing RNA synthesis and that signal transductions induced by the two agents are related. In the cell, cAMP and TPA activate distinct signaling pathways but are otherwise quite related. Both agents trigger signaling by activating serine/threonine kinases, and the transcription factors that are eventually activated by these signals are also related: the cAMP and TPA signals activate CREB/ATF and AP1, respectively, which are highly related transcription factors containing basic/leucine zipper domains, recognize highly similar sequences and can cross-dimerize (see reviews Angel et al. 1991; Delmas et al. 1994). A protein responsible for induced LFB3 mRNA degradation could be phosphorylated and regulated by cAMP-dependent protein kinase as well as by protein kinase C. Alternatively, the two different but related transcription factors may exert their effects at a post-transcriptional step by interacting with the same RNA sequence or RNA-binding protein. It should be remembered that colchicine, cytochalasin B and okadaic acid also reduce LFB3 mRNA (Figure 12) and that these agents do not require protein kinase C to activate AP1 and induce the uPA gene (Lee et al. 1993b; Lee et al. 1994b).
Identification of regulatory sequences in LFB3 mRNA and the corresponding binding proteins should help answer these questions.

We have shown that uPA inducers reduce LFB3 mRNA levels. Is there any physiological significance in this apparent linkage, or is this reverse regulation fortuitous, using very common signaling pathways? uPA is a secreted protease which plays an important role in various extracellular proteolytic processes (reviews Dano et al. 1985; Saksela et al. 1988; Shyu et al. 1989; Kwaan, 1992), but its unchecked expression may have deleterious effects on producing organs or nearby organs (Heckel et al. 1990). As LFB3 is an abundant transcription factor in kidney (De Simone et al. 1991 and P.-A. Menoud unpublished) and is involved in uPA gene regulation, it may have evolved so that the kidney cells use LFB3 as one means to control the level of uPA expression.
5.2 A CBP-independent Mechanism for cAMP Induction of the uPA Gene in LLC-PK1 Cells

5.2.1 Summary
The pig urokinase-type plasminogen activator (uPA) gene has a cAMP-inducible enhancer 3.4 kb upstream of the transcription start site and made up of three protein-binding domains A, B and C. Domains A and B contain a CRE (cAMP response element)-like sequence but require the adjoining C domain for full cAMP responsiveness on a heterologous promoter. A tissue-specific transcription factor, LFB3/HNF1β/vHNF1, binds to the C domain. When the CRE-like sequence was converted to the consensus CRE sequence, mutated AB domains could mediate cAMP induction even in the absence of the adjoining C domain. Conversion of the imperfect LFB3 recognition sequence in domain C to the consensus LFB3 recognition sequence led to a high basal promoter activity without cAMP induction. These results suggest that the imperfect CRE- and LFB3-binding sequences are required for tight coupling of hormonal and tissue-specific regulation. We found that CREB and ATF1 bind to domains A and B and that this binding is enhanced upon phosphorylation by protein kinase A. CBP acts as a coactivator in CREB-dependent cAMP induction. While overexpression of CBP enhanced consensus CRE-mediated cAMP induction, it reduced ABC-mediated induction. 12S E1A associates with the transcriptional adapter proteins, p300 and CBP, and reduces AP1- and CREB-dependent gene expression. We found that E1A repressed the consensus CRE-mediated cAMP induction but showed no effect on the ABC-mediated induction. Interestingly, conversion of CRE-like sequences in the AB domains to the consensus CRE rendered the mutated ABC enhancer sensitive to E1A. Mutation analysis of LFB3 showed that the transactivation domain of LFB3 is required for ABC-mediated induction. Our results further suggest that cAMP induction of the ABC-enhancer differs from that of the consensus CRE by using the tissue-specific transcription factor LFB3 in place of CBP.

5.2.2 Introduction
Regulation of gene expression is achieved by binding of transcription factors to specific sites in the gene. Both distribution of transcription factors in the body and the mechanisms by which they exert their action on the promoter vary greatly (see Calkhoven et al. 1996; Cereghini, 1996). Although there are many different transcription factors, there appear to be even more ways of regulating gene expression. In a cell or in the body, the expression of a given gene is subject to varying spatio-temporal regulation, determined by e.g. cell-type, cell-cycle, developmental stage and
environmental change, including hormonal stimuli. Cooperation between different factors multiplies the possibilities for gene regulation, and it may be the norm that genes are regulated by combinations of multiple cis-acting elements to meet widely divergent demands (see e.g. Ayoubi et al. 1996).

Many proteins are phosphorylated by cAMP-dependent protein kinase (PKA), which initiate various cellular events (reviewed in Walsh et al. 1994). Several transcription factors, including multiple variants of CREB (Yamamoto et al. 1988; Ruppert et al. 1992), CREM (Foulkes et al. 1991) and ATF1 (Rehfss et al. 1991; Liu et al. 1993), have been implicated as nuclear targets for PKA. CREM and ATF1 are highly related to CREB and able to dimerize with it (Hurs et al. 1990; Foulkes et al. 1991). All mediate transcriptional activation by binding to CRE-sites (TGACGTCA). CREB is the best-studied CRE-binding protein and is the basis of current concepts of CRE regulation (reviewed in Montminy et al. 1995). Its phosphorylation by PKA is essential for its activation of transcription (Gonzalez et al. 1989). The nuclear protein CBP binds to the phosphorylated CREB and functions as a PKA-dependent transcriptional coactivator of CREB (Chrivia et al. 1993). p300, a protein highly homologous to CBP, was also shown to bind to phosphorylated CREB with a similar affinity and also to act as a transcriptional coactivator of CREB (Lundblad et al. 1995). The adenoviral oncoprotein E1A binds both CREB and p300 and interferes with their coactivator activity (Arany et al. 1995). CBP serves as a coactivator of many other transcription factors, including c-Jun (Arias et al. 1994), c-Fos (Bannister et al. 1995a), c-Myb (Dai et al. 1996), Sap-1a (Janknecht et al. 1996) and nuclear receptors (Kamei et al. 1996), and acts as an integrator of different signaling pathways. CBP also interacts with TFIIB (Kwok et al. 1994) and thereby enhances transcription.

In most cases, the CRE site is not present in the promoter as an isolated, single copy but usually as a multimer or in close proximity to other cis-acting elements (Fisc et al. 1989; Nitsch et al. 1993). The additional cis-elements may recruit other transcription factors, which are often not ubiquitously expressed, and therefore provide the basis for tissue-specific gene expression (reviewed in Lee et al. 1993). The role of CBP as a coactivator has so far not been studied in the context of enhancers with binding sites for tissue-specific transcription factors other than CRE.

We have studied the urokinase-type plasminogen activator (uPA) gene regulation in LLC-PK1 cells, a cell-line derived from pig kidney epithelia (von der Ahe et al. 1990; Menoud et al. 1993; Marksitzer et al. 1995). In these cells, uPA gene expression is regulated by many independent signaling pathways induced, e.g., by cAMP (von der Ahe et al. 1990), cytoskeletal reorganization (Botteri et al. 1990; Lee et al. 1993), tumor
promoter phorbol esters (Lee et al. 1993) or okadaic acid (Yin et al. 1995a). The cAMP-dependent induction of the uPA gene is cell-specific, as cAMP is unable to induce the uPA gene in U937 (Chakraborty et al. 1994), HeLa (Menoud et al. 1993) or F9 cells (Marksitzer et al. 1995). The pig uPA gene has a cAMP-responsive enhancer located 3.4 kb upstream of transcription initiation site and comprised of three domains A, B and C. Both the A and B domains contain a CRE-like sequence (TGACG), which is essential for cAMP induction, but require the adjoining C domain to confer full cAMP-inducibility on a heterologous promoter (Jans et al. 1987). The C domain is distinct from the A and B domains in that it contains no CRE. We have purified the protein binding to the C domain and found it to be the pig equivalent of LFB3, also known as vHNF1 (Bauhueter et al. 1988) or HNF1β (Menel et al. 1991b), which is highly expressed in kidney (Menoud et al. 1993). LFB3 is related to the liver-specific transcription factor HNF1α (also termed LFB1) and highly enriched in kidney; both factors recognize the same consensus HNF1 sequence, in vivo and in vitro (De Simone et al. 1991). The C-domain sequence contains two imperfect HNF1 recognition sequences (von der Ahe et al. 1990). In the present study, we characterized the ABC-enhancer and the requirements for coupling tissue-specific and hormonal gene regulation. Furthermore, we demonstrated that ATF1 is one of the proteins binding to the A and B domains and showed that the ABC enhancer is regulated in a CBP-independent mechanism. We suggest that LFB3 functionally substitutes for CBP.

5.2.3 Results

5.2.3.1 Cooperation among three Domains in a cAMP-responsive Enhancer

To characterize the ABC enhancer, we introduced a series of mutations (Figure 18A) and cloned the enhancer in front of a luciferase reporter gene with a minimal thymidine kinase-promoter. We examined the cooperation between AB and C domains by inserting between them 5 or 10 nucleotides, corresponding, respectively, to a half or a full turn of the DNA-helix. The insertion of 5 nucleotides (AB5C) reduced the inducibility to about 30% of the wild type (Figure 18B). Insertion of 10 nucleotides (AB10C) reduced the inducibility even further. These results suggest that the specific angular orientation and also the proximity of these proteins, LFB3 and the proteins binding to the AB domains, are important for their functional cooperation in mediating cAMP induction.
**5.2.3.2 Coupling of Hormonal and Tissue-specific Gene Regulation**

The sequences in the ABC domains responsible for cAMP induction are imperfect CRE and HNF1-binding sequences. We examined the significance of their deviation from the consensus CRE and HNF1-binding sequences. When the imperfect CRE sequence was converted to the consensus CRE sequence (Figure 18A), mutated AB domains (termed A*B*) mediated cAMP inducibility without the adjoining C domain. The conversion of the domain C sequence to the consensus HNF1-recognition sequence (termed ABHNF) led to a strong increase in basal promoter activity as well as cAMP inducibility (Figure 19A). To show that this effect depended specifically on LFB3, cotransfection experiments were performed in F9 cells lacking endogenous LFB3. We used the catalytic subunit of PKA to induce these cells since undifferentiated F9 cells
are not responsive to cAMP (Hurst et al. 1990). The results in Figure 19B show that the cotransfection of an LFB3-expression vector had no effect on the ABC enhancer unless there was a cAMP signal but strongly induced luciferase expression from the ABHNF-driven promoter without a cAMP signal. In the ABHNF-enhancer, the imperfect HNF1-binding sequences in the C domain are converted to the consensus HNF1-binding sequences. These results suggest that the imperfect CRE sequences in domains A and B and the imperfect HNF1-binding sequences in domain C are required for tight coupling of hormonal and tissue-specific regulation; the ABC-enhancer is active only in the presence of a cAMP signal and LFB3.

![Graph A](https://example.com/graph_a)

**Figure 19:** Significance of the deviation of protein-binding sequences in the ABC enhancer from respective consensus sequences. A. Different constructs were transiently transfected into LLC-PK1 cells; 16 h later cells were induced by 1 mM Br-cAMP for 6 h and luciferase activity measured. B. pABC-TATA and pABHNF-TATA were transiently transfected into F9 cells with or without expression vectors for the catalytic subunit of PKA (CEV) and LFB3. After 16 h transfection, cells were collected and luciferase activity was measured. For details of the different constructs, see 4.3 and Figure 18A.

5.2.3.3 The ABC-Enhancer does not Mediate TPA-Dependent Gene Induction

The CRE-like elements in the A and the B-domains differ in only one and two nucleotides, respectively, from the consensus AP1 site, TGA(C/G)TCA, also called the TPA response element (TRE). This sequence similarity prompted us to ask if the wild-type ABC enhancer or the mutated ABC enhancer AP1C (see Figure 18A), where CRE-like sequences in the AB domains are converted to the consensus AP1 sequence, could
mediate TPA induction. As shown in Figure 20, TPA treatment led to no more than a 1.5-fold induction from the ABC or AP1C construct. This induction is comparable to the empty vector pTATA and, therefore, not significant. In contrast, a construct with three AP1-binding sites was approximately threefold inducible. Thus the ABC enhancer cannot mediate TPA-induction. Conversion of CRE-like elements into perfect AP1-sites did not make the cAMP-inducible enhancer TPA-inducible. This suggests that LFB3 cooperates specifically with the AB-binding proteins and not with AP1-factors.

![Figure 20: Specificity of LFB3 cooperation.](image)

**Figure 20: Specificity of LFB3 cooperation.** LLC-PK₁ cells were transfected with different reporter constructs and 16 h later induced either with 1 mM Br-cAMP or with 100 ng/ml TPA for 6 h. Cells were then collected and luciferase activity measured. pAP1C-TATA contains three copies of the AP1 sequence from the collagen gene. In the pAP1C-TATA construct, CRE-like sequences were converted to AP1. For details of these constructs, see 4.3. Values are plotted as fold induction (induced over non-induced).

### 5.2.3.4 ATF1 Interacts with the AB-Domains

Several transcription factors have been reported to mediate cAMP-dependent gene activation, of which the best studied so far are CREB and ATF1. We examined the possible involvement of these proteins by testing the effects of antibodies against these molecules in electromobility shift assays using nuclear extracts from LLC-PK₁ cells. As shown in Figure 21A, oligonucleotides of domain A and B sequences gave rise to single bands which could be competed by excess cold oligonucleotide of the same sequence. Inclusion of anti-CREB-specific antiserum in the binding reaction with domain A and B oligonucleotides produced a band slower than the main band. Inclusion of anti-ATF1-specific antiserum produced two slow migrating bands. In contrast, a non-specific antiserum did not change the migration pattern. This suggests that the retarded bands produced by the anti-CREB- and anti-ATF1-antisera are specific and that CREB and
ATF1 can bind both A and B domains. The two retarded bands caused by anti-ATF1-antiserum may indicate different ATF1-containing complexes: an ATF1 homodimer and an heterodimer with another protein.

**Figure 21: Involvement of ATF1 and CREB in ABC enhancer-mediated cAMP induction.** A. Electromobility shift assays. Nuclear extracts prepared from LLC-PK₁ cells were tested for binding to oligonucleotides containing sequences from the somatostatin promoter CRE region or from domains A or B. Radioactive oligonucleotides indicated at the bottom of each panel were incubated without (-) or with nuclear extracts plus anti-ATF1 antibody (α-ATF1), anti-CREB antibody (α-CREB), non-specific antiserum (ns. IgG) or a 100-fold excess of cold oligonucleotide of the same sequence. Reaction products were fractionated in non-denaturing acrylamide (5%) gel electrophoresis and exposed to a PhosphorImager or X-Omat X-ray film with an intensifying screen at -70°C for 4 days for the A- and B-probes and 3 days for the somatostatin CRE-probe. B. Effects of ATF1 overexpression on ABC-enhancer mediated cAMP induction. LLC-PK₁ cells were transfected with pABC-TATA with increasing amounts of the ATF1 expression vector pJ6-ATF1 and 16 h later treated with (■) or without (○) 1 mM Br-cAMP for 6 h. Cells were collected and luciferase activity measured. Values were normalized against those obtained using corresponding amounts of empty expression vector and plotted.
To examine the involvement of CREB and ATF1 in ABC enhancer-mediated induction, cotransfection experiments were performed using expression vectors for CREB and ATF1 in LLC-PK1 cells. Overexpression of ATF1 increased the cAMP-inducibility of the ABC enhancer (Figure 21b), suggesting that ATF1 is in fact involved in the ABC enhancer-mediated cAMP induction. The effect of overexpression of CREB was very different, in that a small amount of the CREB-expression vector resulted in the strong suppression of basal and cAMP-induced luciferase expression (Figure 22). This suppressive effect of CREB was not specific for the ABC enhancer but was also observed with the somatostatin CRE- and SV40 early promoter-driven reporter genes.
Furthermore, CREB-mediated suppression was not cell specific; it was observed in both LLC-PK₁ cells and HeLa cells (data not shown). Thus, CREB overexpression neither confirmed nor contradicted the possible involvement of CREB in the ABC enhancer. A CRE-independent inhibitory effect of CREB has been reported previously (Lemaigre et al. 1993) but the underlying mechanism was not elucidated. Since CREB and ATF₁ can both bind to A and B domains, we postulate that CREB participates with ATF₁ in the ABC-enhancer in the control of cAMP induction.

To date, ATF₁ was found in all cell lines examined (Rehfuss et al. 1991; Pongubala et al. 1995). To test our proposition that ATF₁ is involved in the regulation of the ABC enhancer, we examined ABC enhancer-mediated cAMP induction in NIH 3T3, COS-1, 293 and HeLa cell lines, of which 293 and HeLa have been shown to express ATF₁ (Rehfuss et al. 1991). In all cell lines, the ABC enhancer was inducible when the catalytic subunits of PKA and LFB3 were cotransfected, suggesting that the proteins binding to domains A and B are ubiquitous (Figure 21C).

5.2.3.5 Binding of ATF₁ and CREB to Domains A and B is Regulated by PKA-mediated Phosphorylation

We addressed the role of the phosphorylation of ATF₁ and CREB by PKA. Nuclear extracts from LLC-PK₁ cells were treated with PKA and DNA-binding activity was measured in electromobility shift assays in the presence of specific antisera against CREB and ATF₁ (Figure 23). Treatment with PKA resulted in increased DNA-binding activity of CREB and ATF₁, suggesting that the binding of both proteins is increased upon phosphorylation. In contrast, binding of ATF₁ or CREB to consensus CRE was not affected by PKA-treatment. This result is in agreement with previous reports that asymmetric CREs are only weakly bound by CREB unless they are phosphorylated, whereas symmetrical consensus CREs also have a high affinity for non-phosphorylated forms of CREB (Nichols et al. 1992).
Figure 22: Dose-response of CREB-expression vector. LLC-PK₁ cells were transfected with increasing amount of CREB-expression vector and 1 µg of luciferase reporter plasmid (pABC-TATA, pSSCRE-TATA or pGL2-control).
Figure 23: Increased DNA-binding activity of nuclear extracts from LLC-PK₁ cells by PKA treatment. Nuclear extracts were treated as indicated with or without 100 units of the catalytic subunit of protein kinase A for 90 minutes at 37°C. Afterwards, samples were incubated with or without 2 μg of the indicated antibodies for 4 h at 4°C. Samples were assayed for DNA-binding activity as described in Figure 21. The gels were dried and exposed to X-Omat AR film for 1 day for the somatostatin CRE probe and for 4 days for the domain B probe.
5.2.3.6 The Transactivation Domain of LFB3 is Required for ABC-Enhancer mediated cAMP-Induction

In the albumin promoter, the LFB1 binding site is sufficient to drive liver-specific transcription (Lichtsteiner et al. 1989). The carboxyl-terminal transactivation domain of LFB1 is required to mediate this transactivation. Although the transactivation domains of LFB3 and LFB1 share only 47% homology, the transactivation domain of LFB3 also leads to transcriptional activation of the albumin promoter, but to a lower extent (Ringeisen et al. 1993). In contrast, the C domain in the uPA enhancer, to which LFB3 binds, by itself does not function as a basal enhancer, even with the adjoining AB domains nor does it mediate cAMP induction unless it is linked to the AB domains. Thus, the role of LFB3 differs with the albumin promoter compared with the uPA promoter. We, therefore, asked the question whether the transactivation domain of LFB3 is required for ABC enhancer-mediated cAMP induction. For this, we prepared a series of LFB3 mutants mainly with deletions in the carboxyl-terminal where the transactivation domain is located. To test the activity of these mutants, HeLa cells were used which are devoid of LFB1 and LFB3 (De Simone et al. 1991). As expected, the albumin promoter was active when the full length LFB3 protein was present and when only the dimerization domain at the amino-terminus was deleted (Figure 24A). Deletion of the carboxyl-terminal domain led to a drastic reduction of transactivation activity, confirming that the carboxyl-terminus of LFB3 is required for transactivation of the albumin promoter (Figure 24B). Although, deletion of the amino-terminal dimerization domain was deleterious, such mutants still maintained significant levels of transactivation activity (Figure 24B). When the effects of these mutations on the ABC enhancer were evaluated, the situation was very similar to that with the albumin promoter. Mutants without the carboxyl-terminal showed no mediation of the cAMP signal, whereas a mutant without the dimerization domain still maintained activity though at a reduced level (Figure 24C). This result suggests that the LFB3 transcriptional activation domain is just as essential for ABC-mediated cAMP induction as it is in the context of the albumin promoter.
Figure 24: Requirement of the amino-terminal transactivation domain in ABC-enhancer-mediated cAMP induction. A. Deletion constructs of LFB3. Different constructs A - F were prepared by subcloning various parts of the LFB3 cDNA in the same expression vector used for full-length LFB3 cDNA. The nucleotide positions starting at 150 are indicated on top of the schematic representation. The amino-terminal dimerization domain (DD) is followed by a POU-specific-like domain (POUs), the POU homeodomain (POUhd) and the carboxyl-terminal transactivation domain. The proline/glutamine-rich domain is shown as a box (TA). B. Effects of different LFB3 mutants on the albumin promoter. HeLa cells were transfected with 1 μg of pAlb-luc together with 100 ng of expression vector for various LFB3 mutants. After 20 h transfection, cells were collected and luciferase activity measured. C. Effects of different LFB3 mutants on the ABC-enhancer-mediated cAMP induction. HeLa cells were transfected with pABC-TATA with or without pCEV together with expression vectors for different LFB3 mutants. Assays were done in duplicate and mean values are shown with error bars.
5.2.3.7 A CBP-Independent Mechanism for the ABC-Enhancer mediated cAMP-Induction

We next addressed the question of whether CBP, the coactivator of CREB, is also involved in the ABC enhancer-mediated cAMP induction by performing cotransfection experiments in HeLa cells, which contain low levels of endogenous CBP (J. Arias, personal communication). To our surprise, increasing amounts of CBP led to a strong decrease in the induction of luciferase gene expression from the ABC-enhancer driven promoter (Figure 25B). In contrast, CBP showed positive effects on both induced and basal levels of luciferase gene expression from the somatostatin CRE-driven promoter (Figure 25A), confirming earlier observations that CBP can act as a transcriptional coactivator in the cAMP pathway (Kwok et al. 1994). Interestingly, conversion of the imperfect CRE sequences in the AB domains to the consensus CRE sequences rendered the resulting A*B*C enhancer positively responsive to CBP (Figure 25C). These results strongly suggest that transcriptional activation by the ABC enhancer is mediated by a CBP-independent mechanism.

![Figure 25: CBP-independent mechanism for ABC enhancer-mediated cAMP induction. HeLa cells were transfected with a luciferase reporter construct, pSSCRE-TATA (A), pABC-TATA (B) or pA*B*C (C), with increasing amounts of CBP expression vector pCMV-CBP with or without pCEV. After 20 h of transfection, cells were collected and luciferase activity measured. Assays were done in duplicate and mean values are shown with error bars.]

5.2.3.8 12S E1A has no Influence on the ABC-Enhancer

The adenoviral oncoprotein 12S E1A has been reported to associate with transcriptional adapter proteins, p300 and CBP, thereby interfering with their activities as cofactors in AP1- and CREB-dependent gene expression (Arany et al. 1995). If CBP is involved in the ABC enhancer-mediated cAMP induction, 12S E1A should exert negative effects on the ABC enhancer. We compared the effect of 12S E1A on CRE-and ABC-mediated cAMP induction by transient cotransfection experiments in LLC-PK1 cells. The results in Figure 26 show that 12S E1A appreciably reduced the cAMP inducibility of the somatostatin CRE-linked luciferase gene, while it showed no effect on the ABC-linked luciferase gene. Neither the empty vector nor 13S E1A had a
significant effect. The absence of a 13S E1A effect may depend on the amount of endogenous CREB, ATF1 and CBP, which differs between cell lines (Lee et al. 1996). Interestingly, when CRE-like sequences in the AB domains were converted to the consensus CRE, the resulting A*B*C became sensitive to 12S E1A.

![Graph showing fold induction](image)

**Figure 26: No influence of 12S E1A on ABC enhancer-mediated cAMP induction.** LLC-PK1 cells were transfected with 1 µg of pABC-TATA, pSSCRE-TATA or pA*B*C-TATA together with 0.5 µg of empty vector (pHβApr1) or with expression vectors for 12S E1A or 13S E1A. After 16 h transfection, cells were induced for 6 h with 1 mM Br-cAMP. Mean values are shown with error bars as fold induction (+cAMP/-cAMP).

### 5.2.4 Discussion

The ABC-enhancer of the pig uPA promoter consists of three protein-binding domains and mediates cAMP-induction. The presence of all three domains is required for the full inducibility. We showed previously that LFB3 binds to the C domain and cooperates with the proteins binding to domains A and B (Menoud et al. 1993; Marksitzer et al. 1995). In the present study, we report that ATF1 and CREB also bind to the AB domains. The ABC enhancer was inducible in all cell lines tested when LFB3 was coexpressed, suggesting that the ubiquitous ATF1 and CREB are likely to be the proteins mediating cAMP induction of the ABC-enhancer. We also characterized the ABC enhancer by creation of several mutations. A 5- or 10-nucleotide insertion between domains AB and the LFB3-binding domain C substantially reduced inducibility. Therefore, both close proximity and a certain angular orientation between LFB3 and AB domain-binding proteins are required for their cooperativity. These requirements suggest a physical interaction, but this is most likely very weak, as several attempts at co-
immunoprecipitation using antibodies against CREB and ATF1 failed (data not shown). Another explanation for these requirements is that LFB3 binding induces a slight DNA bending, which could enhance the binding of a protein to the adjacent AB domains. A similar effect was suspected for LFB1, which binds to the same binding site as LFB3, by binding as a homodimer or a heterodimer with LFB3. Binding of the LFB1 dimer induces either a rearrangement of the monomers relative to the DNA or a conformational change of the DNA (Ceska et al. 1993). Due to the high homology of LFB1 and LFB3 in DNA-binding domains, LFB3 is likely to induce a similar change in DNA structure upon binding to the C domain. In this way, LFB3 could facilitate the binding of CREB/ATF1 to the adjacent B domain.

LFB3 functionally interacts with CREB/ATF1. The consensus CRE sequence differs from the TRE sequence only in one nucleotide (Monminy et al. 1986) and the transcription factors binding to TREs, the AP1-factors, are structurally related to CREB/ATF1, having a basic/leucine zipper domain (bZIP) for DNA binding motif (Kourides et al. 1988). However, AP1-factors cannot heterodimerize with CREB (Benbrook et al. 1990) or with ATF1 (Hai et al. 1991b; Masson et al. 1993b). We tested the possible interaction of LFB3 with AP1-factors by converting the imperfect CRE sites into consensus AP1-binding sites. As shown in Figure 20, no significant induction by TPA was detected on such a construct. Therefore, the cooperative role of LFB3 is specific for the cAMP-induction of the ABC enhancer.

The deviation of protein-binding sequences in the ABC-enhancer from the respective consensus sequences is instrumental in ensuring the tight regulation of uPA gene expression in a hormone-dependent and tissue-specific manner. The AB domains alone do not allow cAMP induction, and the C domain alone does not lead to enhanced basal activity. However, if each binding sequence is converted to the consensus element, the A*B* domains without the C domain can mediate cAMP induction and the ABHNF shows considerable basal activity, thus weakening both tissue-specificity and hormone dependency (Figure 19). The most likely explanation for the loss of tissue-specificity with A*B*C is that while CREB/ATF1 binds only weakly to the imperfect CRE sequence, it binds to the consensus CRE with a higher affinity. This postulates enhancement by LFB3 of the affinity of CREB/ATF1 for the AB domains. It is known that PKA-induced phosphorylation influences two aspects of CREB regulation: DNA binding and transactivation. CREB binds to the consensus CRE with a high affinity without being phosphorylated (Nichols et al. 1992), and the role of its phosphorylation lies mainly in recruitment of the cofactor CBP (Chrivia et al. 1993). In fact, we could show that CREB/ATF1 binding to the A and B domains is increased upon
phosphorylation by PKA but that consensus CREs are bound by CREB/ATF1 independently of PKA treatment (Figure 23). The similar results with purified CREB have been described earlier (Nichols et al. 1992). Binding of PKA-treated nuclear proteins to the B oligonucleotide is still much lower than binding of control nuclear proteins to the somatostatin CRE oligonucleotide (Figure 23). It might be that LFB3 is still required for phosphorylated CREB/ATF1 to bind efficiently to CRE-like sequences in the AB domains. Thus, LFB3 is acting as a recruiter of CREB/ATF1 to their binding sites, but at the same time it must be acting as a cofactor in transactivating the transcriptional machinery, because it requires the carboxyl-terminal transactivation domain for activity in the ABC enhancer-mediated cAMP induction. It is less likely that the transactivation domain of LFB3 is involved in the interaction with CREB/ATF1, because in ABC-mediated cAMP induction LFB3 can be replaced by LFBl, whose transactivation domain is least homologous to that of LFB3 compared with other parts of the molecule (see below in Figure 28). We must also accommodate the observation that LFB3 binding to the C domain, unlike to the consensus HNF1-binding sequence, does not enhance basal activity (Figure 19). Due to the deviation from the consensus sequence LFB3 binds less efficiently to the C domain than to the consensus HNF1 site. In this context, it might also be argued that LFB3 bound to the C domain requires interaction with phosphorylated CREB/ATF1 in order to adopt the active conformation required for transactivation activity. Further investigation is necessary to obtain a clear image of the mechanism of cooperativity between LFB3 and CREB/ATF1.

The carboxyl-terminal transactivation domain of the homeodomain protein LFB3 is required for transcriptional activation of the albumin promoter via an HNF1 site (Rey-Campos et al. 1991) and of the ABC enhancer-mediated cAMP induction (Figure 24). This suggests that the transcriptional activation mechanisms of LFB3 as a basal enhancer-binding protein (albumin promoter) and as a coactivator in cAMP induction (the ABC enhancer) are very similar if not the same. The exact molecular mechanism by which LFB3, as well as highly related LFBl, activate the transcriptional machinery has not been elucidated. Similarly, proteins that are contacted by the transactivation domain of LFB3 have so far not been identified. Some homeodomain proteins interact with the basal transcription machinery indirectly through recruiting cofactors which bind to the POU domain (reviewed in Herr et al. 1995). Other homeodomain proteins interact directly with TATA box binding-protein associated factors (TAFs). The homeodomain bicoid has three activation domains which form direct contacts with TAF\(_{n60}\) and TAF\(_{n110}\). The TAF\(_{n110}\) is contacted by the glutamine-rich domain, whereas the alanine-rich domain interacts with TAF\(_{n60}\) (Sauer et al. 1995). It is, therefore, tempting
to speculate that LFB3 containing a proline-glutamine-rich domain could interact directly with one of the TAFs and thereby activate transcription.

The mutant LFB3 that lacks the amino-terminal dimerization domain is less potent than wild-type LFB3 in activating the albumin promoter and in mediating cAMP induction from the ABC-enhancer, but still maintains significant activity on both templates (Figure 24). The conclusion that the dimerization domain of LFB3 is important but not essential for its activity is in agreement with results obtained by others using a similar LFB1 mutant (Nicosia et al. 1990; Tomei et al. 1992). Deletion of the dimerization domain of the LFB1 protein did not lead to a complete loss of DNA-binding activity but to a 50-fold decrease in its affinity for the recognition sequence. In the absence of the dimerization domain, LFB1 can still dimerize on the DNA due to the homeodomain. The homeodomains of LFB1 and LFB3 have a very high homology of 91% (De Simone et al. 1991), suggesting that the regulation of their interaction with DNA is similar. In accordance with this, we have shown that LFB1 can replace LFB3 in ABC-mediated cAMP induction.

Because the transactivation domain of LFB3 is required for transcriptional activation from the ABC enhancer, we addressed the question of how CREB/ATF1 is integrated into this process. CREB is a protein highly related to ATF1 and is the best-studied cAMP-responsive transcription factor. CREB is phosphorylated by PKA at Ser\textsuperscript{133} located in the kinase-inducible domain (KID). Upon phosphorylation, the coactivator CBP can bind to the KID of CREB (Chrivia et al. 1993; Kwok et al. 1994). For binding of CBP to CREB, structural motifs surrounding Ser\textsuperscript{133} are required in addition to the phosphorylated Ser\textsuperscript{133} in the CREB molecule (Chrivia et al. 1993). Truncated CREB with only amino acids 121-151 binds to CBP with a 20-fold lower affinity than the full-length protein, suggesting that sequences outside the region of Ser\textsuperscript{133} are required for interaction of CBP with CREB (Parker et al. 1996). ATF1 differs from CREB mainly at the amino-terminal end and in the amino-terminal part of the KID (Rehfuss et al. 1991; Masson et al. 1993). Furthermore, ATF1 and CREB differ in phosphorylation motifs (Rehfuss et al. 1991). Therefore, it is likely that CBP does not bind as well to ATF1 as it does to CREB (Lee et al. 1996), and that binding of CBP to ATF1 or CREB is regulated through different phosphorylations (Shimomura et al. 1996). Our data suggest that CBP does not act as a coactivator of CREB/ATF1 in the ABC enhancer. The physiological relevance of a CBP-independent transcriptional mechanism remains unclear but a CBP-independent mechanism apparently renders the ABC enhancer independent of other signal transduction pathways. CBP serves as a coactivator of many different transcription factors of distinct signal transduction pathways, including AP1-factors,
Sap-1a, c-Myb and nuclear receptors for glucocorticoids and thyroids (Arias et al. 1994; Bannister et al. 1995; Dai et al. 1996; Janknecht et al. 1996; Kamei et al. 1996). Since the amount of CBP is usually limiting in cells, transcriptional activation by CBP depends on the ability of transcription factors to recruit CBP for coactivation. In this way, CBP negatively integrates different signaling pathways. CBP-independent transcriptional activation of the ABC enhancer after cAMP-stimulation may be important for strong activation of the uPA-promoter in response to cAMP, independent of the TPA-signal transduction pathway. Therefore cAMP-dependent uPA gene expression does not compete with the TPA-dependent uPA gene activation by the PEA3/AP1-site located at -2 kb (Lee et al. 1993; Nagamine et al. 1996).

Coupling of cAMP induction with tissue-specific regulation has also been described for several enhancers, such as those in the phosphoenolpyruvate carboxykinase (PEPCK) and the tyrosine aminotransferase promoters (Nitsch et al. 1993; Roesler et al. 1995; Roesler et al. 1996, reviewed in Lee et al. 1993). In many enhancers, the cAMP-response is not mediated by one single CRE but by more than one cis-element. In these promoters, either multiple CREs are present or a CRE interacts with a factor bound to another cis-element (reviewed in Lee et al. 1993). In the latter case, the second factor can provide tissue specificity. In the case of the PEPCK gene, transcription is strongly induced after treatment of liver cells, but not kidney cells, with agents that augment cAMP concentration. Liver-specific gene expression of PEPCK is ensured by cooperation between different transcription factors. cAMP responsiveness is provided by CREB binding to an imperfect CRE sequence, whereas tissue specificity is mediated by three sites, two of which are recognized by C/EBPα and another by AP-1. The tissue-specific regulation of the PEPCK gene is achieved by cooperation of two regions in the promoter, one region containing an imperfect CRE sequence and the other containing three C/EBP binding sequences and an AP1-binding site. All five proteins must bind to their respective sites for full promoter activation, although the physical nature of cooperation among them still remains to be studied (Roesler et al. 1996). In the case of the somatostatin gene, which contains the prototype consensus CRE sequence, a region of at least 20 nucleotides immediately upstream of the palindromic CRE is required for full cAMP responsiveness (Montminy et al. 1986). This suggests that not only imperfect but also perfect palindromic CREs require adjacent protein-binding sites in order to mediate cAMP-responsiveness in the physiological context. CBP is a coactivator of CREB when studied in the context of the somatostatin CRE. Multiple contacts between the transcription factors and the basal transcription machinery are required to trigger transcription. For an isolated CRE, CREB and CBP form contacts to the basal transcription factors (Ferreri et al. 1994; Kwok et al. 1994). Because most CREs are not
present as single elements but in combination with other CREs or binding sites for tissue-specific transcription factors, these additional binding sites help to form multiple contacts with the basal transcription factors. Thus, it is likely that the function of CBP can be substituted by another transcription factor. In the case of the ABC enhancer in the uPA gene, LFB3 may substitute for the CBP function by contacting the basal transcription machinery with its transcriptional activation domain. The treatment of cells with cAMP leads to increased binding of CREB/ATF1 to the AB domains, which might affect the nature of and activate LFB3 binding to the adjacent site (see above). The analysis of other enhancers in which tissue-specific transcription factors and CREB/ATF1 synergize in the transcriptional activation may reveal further CBP-independent gene regulation.
5.3 The ABC-Enhancer is Regulated Differently in F9 Cells than in LLC-PK<sub>1</sub> Cells

The pluripotent embryonic teratocarcinoma F9 cells provide an attractive model to study cAMP-induction of gene expression (Montminy et al. 1986; Weigel et al. 1990), because these cells are devoid of active CREB and ATF1 by an unknown mechanism (Masson et al. 1992; Masson et al. 1993). Treatment of these cells with retinoic acid for several days leads to the differentiation of these cells into different phenotypes depending on the presence of cAMP (Strickland et al. 1987). Undifferentiated F9 cells do not contain LFB3, but in differentiated F9 cells LFB3 protein is expressed (De Simone et al. 1991). Therefore, to induce the ABC-enhancer in undifferentiated F9 cells, expression vectors for LFB3 and protein kinase A (CEV) have to be cotransfected.

5.3.1 Comparison between LLC-PK<sub>1</sub> and F9 cells

Since F9 cells are often used to study the cAMP-pathways, we hypothesized that results obtained in F9 cells could contribute to the elucidation of the mechanism by which the ABC-enhancer is regulated. Given that many genes are differently regulated in different cell lines, we initially compared F9 cells with LLC-PK<sub>1</sub> cells for the cAMP-inducibility of various constructs with mutations in the ABC-enhancer. The results, shown in Figure 27, are described in detail in the following paragraphs.

5.3.2 The difference between two ABC-Constructs

The ABC-enhancer was inserted into the pTATA-luc immediately upstream of the TATA-box (see Materials and Methods in chapter 4.3). Two constructs were obtained; they differ only in the stuffer sequences shown below in italics (only the region around 5' end of the ABC-enhancer is shown):

\[
\begin{align*}
pTATA-insertion site & \quad \text{stuffer} & \quad \text{ABC-enhancer 5'end} \\
ABC-construct & \quad 5'-\text{CTGCAGGTCG-GGGAGGrACC-CTGTGCCTGA-3'} \\
ABCRM-construct & \quad 5'-\text{CTGCAGGTCG-AC-CTGTGCCTGA-3'}
\end{align*}
\]

The two constructs behave similarly when tested in LLC-PK<sub>1</sub> cells (see Figure 27a). To our surprise, however, when assayed in F9 cells a big difference was detected (see Figure 27b). The ABC-construct was more strongly inducible by CEV than the ABCRM-construct when LFB3 was cotransfected. The sequence causing the difference has no homology to any known transcription factor binding site.
5.3.3 Cooperativity at the ABC-Enhancer

We examined if the cooperativity among the three domains is similar in both cell lines (see Figure 27). In LLC-PK1 cells, all three domains A, B and C have to be adjacent to mediate cAMP-induction. Constructs with mutations in domain A (aBC) or domain B (AbC) lead to only 20% inducibility compared to ABCRM (see Table 5-1). Similarly a construct with mutated domain C (ABc) leads only to about 15%
inducibility. Therefore in LLC-PK₁ cells, for full induction of the ABC-enhancer all three domains are required and mutation of one of the three domains leads to about 80% reduction in cAMP-inducibility.

In F9 cells, however, the situation was rather different. Like in LLC-PK₁ cells, the highest induction was detected when the ABCRM-construct was used, but the influence of mutation in any of the three domains affected the inducibility less pronounced: The aBC and AbC were able to mediate 50% of the cAMP-inducibility compared to ABCRM (see Table 5-1). Thus, the cooperativity among the three domains is less strong. Since this result is in clear contrast to the situation in LLC-PK₁ cells, we think that in F9 cells the ABCRM-construct is not regulated in the same manner as in LLC-PK₁ cells. Thus, cell-specific differences contribute to a different regulation of the ABC-enhancer in F9 cells than in LLC-PK₁ cells.

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<td>aBC</td>
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<tr>
<td>AbC</td>
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<td>ABc</td>
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<td>ABCRM</td>
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Table 5-1: Cooperativity of the ABC-enhancer, a cell-specific phenomenon. Analysis of the data shown in Figure 27: The inducibility of some constructs with mutations in the ABC-enhancer was quantified and compared to the inducibility of the ABC-enhancer, which was set to 100%.

5.3.4 LFB1 is able to Induce the ABC-Enhancer like LFB3

LFB1 is related to LFB3 and both proteins can heterodimerize with each other. Since both proteins recognize the same binding site, we wondered if for the induction of the ABC-enhancer specifically LFB3 is required or if LFB1 also can functionally replace LFB3.

Using an expression vector for LFB1, we detected similar effects as observed by LFB3, suggesting that both proteins have the same potential to mediate cAMP-responsibility at the ABC-enhancer (see Figure 28).
5.3.5 Inducibility Observed on AAC and BBC

As shown in Figure 27, in LLC-PK₁ cells the constructs AAC and BBC constructs are not as strongly inducible as the ABCRM constructs (60% and 70%, respectively). This suggests that the proteins binding to A and B are different from each other. In F9 cells, the AAC and BBC constructs evoked similar effects on the inducibility (60% and 65%, respectively) as in LLC-PK₁ cells when compared to the ABCRM construct.

5.3.6 Does LFB3 Expression for 15 Hours lead to Differentiation of F9 Cells?

In undifferentiated F9 cells, LFB3 is not expressed, but becomes expressed upon treatment with retinoic acid (De Simone et al. 1991). Differentiation of these cells induced by treatment with retinoic acid leads to cAMP-responsiveness. The ABC-enhancer was not induced by cAMP-treatment in undifferentiated F9 cells, only when CEV was cotransfected a significant level of luciferase activity was measured (see Figure 29). In contrast in F9 cells differentiated by retinoic acid treatment, addition of cAMP led to an as strong induction of the ABC-enhancer as when CEV was cotransfected. This effect was detectable also in the absence of exogenously added LFB3. When LFB3 was additionally cotransfected, the cAMP- and the CEV-inducibility was stronger most likely due to low levels of endogenous LFB3 in the differentiated cells.

These results indicate that cotransfection of LFB3 does not lead to differentiation of the F9 cells because no cAMP-responsiveness was observed in the undifferentiated F9 cells transfected with LFB3 (see Figure 29).
Figure 29: The ABC-enhancer is inducible in RA-differentiated F9 cells by Br-cAMP. F9 cells were treated with 0.1 μM retinoic acid (RA) for 5 days and subsequently transfected with 1 μg pABC-TATA, 1 μg CEV and 100 ng RSV-FPCB (LFB3 expression vector). When cells were to be induced by cAMP, 100 mM Br-cAMP was added to the cells for 6 hours. Cells were harvested and luciferase activity was measured. UF9 = undifferentiated F9 cells, DF9 = differentiated F9 cells (see Strickland et al. 1987).

In the differentiated cells, the ABC-enhancer is more strongly inducible by CEV and LFB3 than in undifferentiated F9 cells, suggesting that an activating factor which is absent in undifferentiated is present in differentiated cells.

5.3.7 Effect of CREB and ATF1 Overexpression in F9 Cells

We confirmed earlier results that a luciferase construct driven by the somatostatin CRE can be induced in undifferentiated F9 cells only upon cotransfection of expression vectors of protein kinase A (CEV) and of CREB or ATF1 (Montminy et al. 1986; Rehfuss et al. 1991) (see Figure 30). We analyzed the effect of CREB or ATF1 overexpression on the ABC-enhancer. The ABC-enhancer (pABC-TATA) was strongly inducible when CEV and LFB3 was cotransfected. If CREB or ATF1 was overexpressed a reduction in inducibility was observed. A similar reduction in inducibility of pABC-TATA and pA*B*C-TATA was observed in LLC-PK1 cells when CREB was overexpressed.
Figure 30: Effect of CREB- and ATF1-overexpression in UF9 cells. pSSCRE-TATA, pABC-TATA and \( \text{pA}^*\text{B}^*\text{C}-\text{TATA} \) were tested for their transcriptional activity in the presence or absence of CREB and ATF1. Undifferentiated F9 cells were transfected with 1\( \mu \)g luciferase construct with 1\( \mu \)g pSV-CREB or pSV-ATF1 expression vector and 1\( \mu \)g CEV where indicated. For the constructs pABC-TATA and \( \text{pA}^*\text{B}^*\text{C}-\text{TATA} \), 1\( \mu \)g pRSV-FPCB was additionally cotransfected.

5.3.8 Discussion

Our data suggest that in F9 cells the ABC-enhancer is not similarly regulated as in LLC-PK\(_1\) cells. The cooperativity among the three domains is different in the two cell lines, suggesting that cell-specific factors present in LLC-PK\(_1\) cells are missing in F9 cells. However, when F9 cells are treated by retinoic acid and induced to differentiate this factor may become expressed.

It was postulated that F9 cells contain only inactive CREB and inactive ATF1. Although these proteins are present in undifferentiated cells in a much higher quantity than in the differentiated cells, they are inactive by an unknown mechanism (Masson et al. 1993a). Therefore, overexpression of CREB or ATF1 was demonstrated to be necessary for induction of a somatostatin CRE construct fused to a CAT-reporter gene...
We present data which confirm that overexpression of CREB or ATF1 in undifferentiated F9 cells exerts a positive effect on the construct with the somatostatin CRE when CEV was cotransfected (see Figure 30). An induction of the somatostatin CRE construct without CREB or ATF1 expression was still detectable which may be weak in absolute terms but strong in terms of fold induction. An opposite effect was observed on pABC-TATA and pA*B*C-TATA. Strong induction of both constructs was measured without cotransfection of CREB or ATF1. When cotransfected, they even led to reduced inducibility. This effect could be due to a negative effect exerted of CREB and ATF1 on the LFB3 expression vector or also on the empty vector pTATA. Such a negative effect of CREB and ATF1 was already described for various promoters independent of the presence of CRE-sites (Lemaigre et al. 1993).

In conclusion, several data support the fact that undifferentiated F9 cells are not the ideal background to study the regulation of the ABC-enhancer.
6. GENERAL DISCUSSION

In this thesis different aspects of the regulation of the uPA gene expression by cAMP were presented. In this chapter a short overview of the cAMP-dependent regulation of the ABC-enhancer will be presented and some aspects will be discussed which have not been discussed before. An outline will be given which additional experiments that would be interesting to perform.

6.1 Model for the Transcriptional Regulation of the ABC-Enhancer

The activation of a cAMP-responsive enhancer located 3.4 kb upstream of the transcription initiation site of the uPA gene requires the cooperation of three adjacent protein-binding sites. To these three sites CREB/ATF1 and LFB3 bind and lead to transactivation. Since the binding of LFB3 to the ABC-site is required, a decrease in the amount of LFB3 protein reduced the inducibility of the uPA gene by cAMP. The amount of LFB3 is reduced after an increase in cAMP and other inducers of the uPA gene, including TPA, by reducing the stability of its mRNA. This effect of cAMP and TPA raises the interesting possibility of a cross-talk between cAMP and the other signal transduction pathways involved in the induction of the uPA gene. In this way, the reduction of LFB3 exerts a negative feedback regulation which prevents a sustained high expression of the uPA gene. The uPA gene is induced by the binding of CREB/ATF1 and LFB3 to the ABC-site. So far phosphorylated CREB has been shown to recruit CBP and to initiate the transcriptional activation. The binding of transcription factors to the ABC-site leads to transcriptional activation without CBP, but requires LFB3 and its transactivation domains, suggesting that LFB3 can substitute for CBP. A model for this hypothesis is shown in Figure 31. Data supporting this model are presented in this thesis.

6.2 Mechanism of Transcriptional Activation

In the model presented above we suggest that CREB and ATF1 are involved in the transcriptional activation of the ABC-enhancer and that this activation occurs through a CBP-independent mechanism.

6.2.1 Confirmation of CREB- and ATF1-Involvement in the ABC-Enhancer

The data obtained about the involvement of CREB and ATF1 in the ABC-enhancer were not compelling. ATF1 had a positive effect on the ABC-enhancer when it was cotransfected with increasing amounts of the ATF1 expression vector. In the same experiment, CREB repressed the activity of the promoter construct due to a general
repressive effect of CREB not only on cAMP-regulated but also on non cAMP-regulated promoters. Antisense expression of CREB and/or ATF1 or expression of dominant negative CREB or ATF1 mutant should confirm the role of CREB/ATF1 in the regulation of the ABC-enhancer.

6.2.2 Does LFB3 Bind to CREB or ATF1?

Insertion of 5 or 10 nucleotides between domains B and C greatly reduces the inducibility of the ABC-enhancer by cAMP. This result indicates that CREB/ATF1 has to be close to LFB3 and that these proteins might interact with each other. This hypothesis could be tested in pull-down assay or with recombinant proteins in gel-shift analysis experiments. An interaction between CREB/ATF1 and LFB3 could have major implications for the CBP-independent transcriptional activation mechanism (see below).

Figure 31: Comparison of the proposed mechanisms of cAMP-induced transcriptional activation of the prototype somatostatin promoter and the ABC-enhancer of the pig uPA gene. In the somatostatin promoter a consensus CRE-site is present and CREB is constantly bound to this site. CREB contacts TAF110 in the TFIID -complex with its Q2-domain. PKA phosphorylates CREB and thereby leads to recruitment of CREB-binding protein (CBP) to CREB. CBP interacts through the N-terminus with TFIID. These multiple contacts lead to transcriptional activation of the somatostatin gene. In contrast, the ABC-enhancer uses a CBP-independent mechanism for gene activation. Due to non-consensus CRE-sites, the binding affinity of CREB/ATF1 to domains A and B is low but is high after phosphorylation by PKA. CREB/ATF1 contacts TFIID with its Q2-domain. The tissue-specific transcription factor LFB3, which is not phosphorylated by PKA, binds only weakly to the C-domain. LFB3 contacts the TFIID complex and thus functionally substitutes for CBP.
6.2.3 Does ATF1 Interact with CBP?

No reports about the possible interaction between ATF1 phosphorylated by PKA and CBP exist in the literature. So far only one publication describes an interaction between CBP and CaMK II -phosphorylated ATF1 (Shimomura et al. 1996). In the introduction of this thesis I reported that the CBP-binding site in CREB is not highly conserved in ATF1. ATF1 also has several different phosphorylation motifs than CREB. It would therefore be interesting to study \textit{in vitro}, using recombinant proteins, whether CBP can bind to PKA-phosphorylated ATF1 equally well as to CREB. If a difference in CBP-binding affinity is observed between CREB and ATF1, then this result would support the involvement of ATF1 in the activation of the ABC-enhancer. However to address the functional significance of such a lack in CBP-binding to ATF1 is very difficult as cell lines lacking CREB or ATF1 do not exist. If CBP can bind equally well to both ATF1 and CREB, then recombinant LFB3 should be included in the binding assay to test whether it can interfere with the binding between CBP-CREB or CBP-ATF1. It is possible that LFB3 binds to CREB/ATF1 and thereby prevents binding of CBP to CREB/ATF1.

6.2.4 To which TAFs Binds LFB3?

As outlined in the introduction to this thesis (see 3.4.1), it is not known with which TAFs LFB3 interacts with its activation domain. By analogy with bicoid, which contacts TAF110 via its glutamine-rich domain (Sauer et al. 1995), it is tempting to speculate that LFB3 might contact TAF110 via its proline-glutamine-rich domain. Interestingly CREB also contacts TAF110 via its glutamine-rich domain Q2 (Ferreri et al. 1994). Thus it is possible that LFB3 and CREB/ATF1 contact TAF110. This would offer another explanation for why domains B and C have to be adjacent for the cooperative induction of the ABC-enhancer by cAMP.

6.2.5 Does the ABC-Enhancer exist in species other than pig?

Until now, the ABC-site has only described in the pig uPA promoter and it has not been found in mouse. In the human uPA promoter this region has not yet been sequenced. It is therefore difficult to speculate about the significance of the ABC-site in the expression of uPA in kidneys. Several experiments indicate that the cAMP-dependent induction of the uPA-gene requires additional sequence elements which are scattered throughout the promoter (von der Ahe et al. 1990; Lee et al. 1994 and R. Marksitzer, unpublished data).
6.3 The ABC-Site is a Prototype Enhancer for Coupling the Tissue-specificity and Hormonal Regulation of a Gene

Gene regulation by cAMP often involves the combined action of a tissue-specific transcription factor with CREB/ATF1 (reviewed in Lee et al. 1993). The reports that deal with the mechanism by which CREB induces transcriptional activation are so far either obtained in vitro (Ferreri et al. 1994) or on promoters that do not contain the tissue-specific transcription factor binding site (Montminy et al. 1995). In this thesis, the question of how the ABC-enhancer combines tissue-specificity and cAMP-dependent gene regulation was addressed. The ABC-enhancer was observed using a CBP-independent mechanism for its transcriptional activation by cAMP. This phenomenon has not yet been described for a cAMP-regulated gene. It may be that other enhancers are also regulated in a similar CBP-independent manner. The recruitment of the cofactor CBP to CREB may be inhibited by neighboring factors, most likely tissue-specific transcription factors. Additional studies on cAMP-dependent enhancers which combine tissue-specificity and cAMP-dependent gene regulation may show a similar CBP-independent mechanism. Therefore it may be that the ABC-enhancer could be the prototype enhancer combining both tissue-specific gene regulation and cAMP-dependent gene regulation with non-consensus protein binding sites.
### 7. ABBREVIATIONS

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<th>A</th>
<th>adenine</th>
<th>GSK</th>
<th>glycogen synthase kinase</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
<td>GST</td>
<td>glutathione S-transferase</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>ARE</td>
<td>AU-rich element</td>
<td>H89</td>
<td>N-2-(p-bromocinnamyl-amino)ethyl-5-isoquinoline sulfonamide</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
<td>HNF1</td>
<td>hepatocyte nuclear factor 1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
<td>IBMX</td>
<td>isobutylmethylxanthine</td>
</tr>
<tr>
<td>BCD</td>
<td>bicoid</td>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>bp</td>
<td>base pair</td>
<td>KID</td>
<td>kinase-inducible domain</td>
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<tr>
<td>Br-cAMP</td>
<td>8-bromo-cAMP</td>
<td>l</td>
<td>liter</td>
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<tr>
<td>Bt2cAMP</td>
<td>dibutyryl-cAMP</td>
<td>LFB3</td>
<td>liver-specific factor B3</td>
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<td>C</td>
<td>cytosine</td>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
<td>NTR</td>
<td>N-terminal region</td>
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<tr>
<td>CaMK</td>
<td>Ca(^{2+})- and calmodulin-dependent protein kinase</td>
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<td></td>
</tr>
<tr>
<td>cAMP</td>
<td>3',5'-cyclic adenosine monophosphate</td>
<td></td>
<td></td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
<td>PA</td>
<td>plasminogen activator</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
<td>PAI</td>
<td>plaminogen activator inhibitor</td>
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<td>CRE</td>
<td>cAMP response element</td>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>CREB</td>
<td>CRE-binding protein</td>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>CREM</td>
<td>CRE modulator</td>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>DcoH</td>
<td>dimerization cofactor H</td>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>DF9</td>
<td>differentiated F9 cells</td>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
<td>RTS</td>
<td>Rubinstein-Taybi syndrome</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
<td>T</td>
<td>thymine</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td>TAF</td>
<td>TBP-associated factor</td>
</tr>
<tr>
<td>DRB</td>
<td>5,6-dichloro-1-β-D-ribofuranosylbenzimidazole</td>
<td>TBP</td>
<td>TATA-binding protein</td>
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<td>DTT</td>
<td>dithiothreitol</td>
<td>TGF-β</td>
<td>transforming growth factor β</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
<td>tPA</td>
<td>tissue type-plasminogen activator</td>
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<tr>
<td>ERG</td>
<td>early response gene</td>
<td>TRE</td>
<td>TPA response element</td>
</tr>
<tr>
<td>ECS</td>
<td>fetal calf serum</td>
<td>UF9</td>
<td>undifferentiated F9 cells</td>
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<td>FPA, B, C</td>
<td>footprint A, B, C</td>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
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<td>FPCB</td>
<td>FPC-binding protein (LFB3 homologue)</td>
<td>uPAR</td>
<td>uPA-receptor</td>
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<td>G</td>
<td>guanine</td>
<td>UTP</td>
<td>uridine triphosphate</td>
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<td></td>
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<td>UTR</td>
<td>untranslated region</td>
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<td></td>
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<td>VP</td>
<td>vasopressin</td>
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Abbreviations for amino acids used in the single-letter code:

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<td>C</td>
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<td>Glutamic acid</td>
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<td>Histidine</td>
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<tr>
<td>R</td>
<td>Arginine</td>
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<tr>
<td>S(*)</td>
<td>Serine (phosphorylated)</td>
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<td>T(*)</td>
<td>Threonine (phosphorylated)</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
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<tr>
<td>Y(*)</td>
<td>Tyrosine (phosphorylated)</td>
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Abbreviations for nucleotide sequences used in the single-letter code:

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<td>Thymidine</td>
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<td>B</td>
<td>C, G or T</td>
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<tr>
<td>X</td>
<td>N - A, C, G or T</td>
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CH- 4002 Basel, Switzerland
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fax: +41 61 697 39 76
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1988-1992 Eidgenössische Technische Hochschule ETH Zurich (Federal
Institute of Technology); Diploma in Biology
1993-1996 Ph.D. program at the Eidgenössische Technische Hochschule Zurich
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Academic Qualifications
1989 1. Intermediate exams at the ETH in Zurich
1990 2. Intermediate exams at the ETH in Zurich
1992 Diploma exams at the ETH in Zurich
1992 Diploma work in the group of Prof. Dr. Hanns Moehler at the
pharmacological department of the University of Zurich; Title of
thesis: GABAA-receptors in the CNS: Immunological
characterization of subtypes containing the α2-subunit.
1993-1996 Ph.D. program at the ETH in Zurich; Graduate work at the Friedrich
Miescher Institute, Basel, Switzerland. Title of thesis: Mechanism
for cAMP-induced, tissue-specific regulation of the urokinase-type
plasminogen activator gene in kidney epithelial cells.
Supervisors are Dr. Y. Nagamine (FMI) and Prof. Dr. H. M.
Eppenberger (ETH).

Languages German, English, French, Latin, Ancient Greek
9.1 Publications


In Preparation

1.) D'Orazio, D., Besser, D., Marksitzer, R., Stacey, K., Hume, D., Nagamine, Y.: A far upstream PEA3/AP1 element is involved in induction of the urokinase-type plasminogen activator gene by different inducers.


Meetings attended

FEBS Meeting 1995
Marksitzer, R., Stief, A., Menoud, P.A., Nagamine, Y.: Role of LFB3 in cAMP regulation of the uPA gene in LLC-PK<sub>1</sub> kidney cells.

Keystone Symposium 1996
Marksitzer, R., Menoud, P.A., Nagamine, Y.: CREB-independent mechanism for the cAMP regulation of the uPA gene in LLC-PK<sub>1</sub> kidney cells.
10. ACKNOWLEDGMENTS

I would have never been able to complete this work without the help, the ideas and the encouragement of many people, to whom I'm very grateful.

Special thanks are due to Dr. Yoshikuni Nagamine who offered me the opportunity to work on this interesting project. He was always willing to discuss problems and always supported me in my work. I appreciate that he never hesitated in pointing out the crucial questions raised by the work while allowing me the scientific freedom to address the questions that I considered to be most important. His knowledge of the field greatly contributed to the development of this project. I hope that he considers the years I spent in his lab as fruitful as I consider them.

I am also very grateful to Prof. Dr. Hans M. Eppenberger of the ETH Zurich, who agreed to act as my supervisor and who often invited me to discuss my project with him and his group.

I was greatly assisted in this project by the work done previously by Drs. Dietmar von der Ahe and Pierre-Alain Menoud. My debt to them is considerable but also to Dr. Aribert Stief, whose work laid the basis for a part of this project and whose jokes and discussions were always refreshing.

Thanks go also to all the members of Yoshi’s lab: Mahmoud El-Shemerly, José Pedro Irigoyen, Magdalena Koziczak, Dr. Christina Kunz, Lilian Montero and Dr. Rika Nanbu. I am also very grateful to Mazin Soubt and Birgitta Kiefer for their assistance with this project. Special thanks are due for the uncountable cells Birgitta transfected for me and for her help during the whole project. Mazin’s enthusiasm and interest in our collaboration accelerated the identification of the proteins involved in the ABC-enhancer and who performed more shifts than we anticipated would ever be necessary. I'm also very thankful for the collaboration with Daniel D’Orazio and Dr. Daniel Besser, who never hesitated to discuss and comment on my results and for his hospitality in New York.

I also want to acknowledge other members of the FMI who contributed to this work: Dr. Carl D. Nager for helping me with the computer facilities and for having been open for many discussions; Dr. Edward Oakeley for critically reading this manuscript and attempting to correct my English and to the secretaries who helped me very efficiently with the administrative issues.

Furthermore I want to thank all my friends outside of the FMI, who not only showed an interest in the science, but also reminded me of the life apart from science.

I am also very grateful and sincerely indebted to my parents. Special thanks are due to them for letting me study what I wanted and for supporting me during difficult periods. I appreciated a lot that they were always open for discussions and that they encouraged me to undertake this project.