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Analysis of the molecular mechanisms underlying the activity of the Ets-1/USF-1 transcription factor complex on the HIV-1 LTR

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Ulrich Mayer

Summary

To assure cell type specific gene transcription cells have developed strategies to control the transcription of a large number of genes with a limited number of transcription factors. This is achieved by combinatorial control in which a complex array of transcription factors regulates promoters and enhancers. Recognition of regulatory elements is governed by both protein-DNA and protein-protein interactions. Many transcription factors can engage in multiple protein-protein interactions that form larger complexes required for adequate gene expression. In this PhD thesis I will present results that illuminate the multifaceted interplay between the transcription factors Ets-1 and USF-1. The ETS proteins act synergistically with a variety of other transcription factors to regulate many cellular and viral promoters and enhancers. Transcription of human immunodeficiency virus 1 (HIV-1), integrated into the host cell genome, also depends on the concerted action of cellular and viral transcription factors recruited to the HIV-1 long terminal repeat (LTR). The cellular transcription factors Ets-1 and USF-1 have been shown to form a complex on adjacent DNA binding sites present in the distal enhancer of the HIV-1 provirus and to cooperate in DNA binding and transactivation. DNA binding of Ets-1 is governed by autoinhibition that is exerted by two distinct inhibitory modules situated N- and C-terminally to the ETS DNA binding domain.

The objective of my thesis project was to unravel the molecular mechanisms that govern the cooperation between Ets-1 and USF-1. I could show that USF-1 interacts with the C-terminal autoinhibitory module of Ets-1 and that this interaction is required to relieve autoinhibition of Ets-1 DNA binding. Reciprocally DNA binding by USF-1 is also facilitated by interaction with Ets-1. Furthermore, I provide evidence that synergistic transactivation by Ets-1 and USF-1 is not only the consequence of increased DNA binding potential but of additional cooperative mechanisms that affect transactivation function itself. I could reveal a novel mechanism of transcription factor

cooperativity by showing that the C-terminal autoinhibitory module of Ets-1 can directly activate transactivation capacity of USF-1. In addition, I show that the transcriptional cofactor CBP is implicated in the mediation of Ets-1/USF-1 cooperativity. CBP interacts physically with both transcription factors and is required for synergistic transactivation. I could map the domain in USF-1 necessary for interaction with CBP to a stretch of 22 amino acids. Deletion of this domain abolishes both transactivation capacity of USF-1 on the HIV-1 LTR reporter and cooperativity with Ets-1.

Together, these data provide new insights into the molecular mechanisms underlying Ets-1/USF-1 cooperativity. They indicate that transcription factor interaction results in significant conformational changes that affect both DNA binding and transactivation function of the complex. The example of Ets-1 and USF-1 could serve as a model for the hypothesis that transcription factors do not act as individual entities but that their functionality is only revealed in the complex with other partner molecules, similar to other multiprotein machineries in the cell.

Analyse der molekularen Mechanismen die der Aktivität des Ets-1/USF-1 Transkriptionsfaktorkomplexes am HIV-1 LTR zugrunde liegen

Ulrich Mayer

Zusammenfassung

Im Laufe der Evolution haben multizelluläre Organismen Strategien entwickelt, um mit einer limitierten Anzahl von Transkriptionsfaktoren die Expression zellspezifischer Gene zu gewährleisten. Diese basieren auf dem Prinzip der kombinatorischen Kontrolle, bei der ein komplexes Zusammenspiel von verschiedenen Transkriptionsfaktoren an Promotor- und Enhancerregionen die Genexpression reguliert. Dabei spielen neben der sequenzspezifischen Bindung von Transkriptionsfaktoren an die DNA auch Bindungen, die die Transkriptionsfaktoren untereinander oder mit weiteren regulatorischen Proteinen eingehen, eine entscheidende Rolle. Auch die Transkription des in das Wirtszellgenom integrierten humanen Immundefizienz Virus (HIV) hängt von der konzertierten Aktion viraler und zellulärer Faktoren ab. Diese binden an spezifische Erkennungssequenzen, die sich in den „long terminal repeats“ (LTR) des HIV-Provirus befinden. Es ist bekannt, dass die zellulären Transkriptionsfaktoren Ets-1 und USF-1 an zwei benachbarte Bindestellen in der Enhancerregion des LTR binden und bei der DNA-Bindung und Transaktivierung miteinander kooperieren.

Das Ziel meiner Promotionsarbeit war es die molekularen Mechanismen aufzudecken, die der Kooperation zwischen Ets-1 und USF-1 zugrunde liegen. DNA-Bindung von Ets-1 wird durch einen autoinhibitorischen Mechanismus reguliert. Hierfür sind zwei inhibitorische Module verantwortlich die sich N- und C-terminal von der DNA-Bindungsdomäne befinden. Ich konnte zeigen, dass USF-1 mit dem C-terminalen, inhibitorischen Modul von Ets-1 interagiert. Durch diese Interaktion hebt USF-1 die Autoinhibition von Ets-1 auf. Darüber hinaus wird auch die DNA-Bindung von USF-1 durch das Wechselspiel mit Ets-1 stimuliert. Desweiteren konnte ich zeigen, dass die synergistische Transaktivierung durch diese beiden Faktoren nicht nur auf einer verbesserten DNA Bindung und somit einer erhöhten Präsenz am

Enhancer beruht. Ich konnte einen neuen Mechanismus aufdecken der zur kooperativen Transaktivierung durch die beiden Transkriptionsfaktoren beiträgt, wobei das C-terminale, autoinhibitorische Modul von Ets-1 direkt das Aktivierungspotential von USF-1 erhöht. Weitergehend konnte ich nachweisen, dass der aktivierende Kofaktor CBP/p300 eine erhebliche Rolle bei der kooperativen Transaktivierung durch den Ets-1/USF-1 Komplex spielt. Hierbei ist die Interaktion von CBP mit USF-1 von besonderer Wichtigkeit. Im Rahmen meiner Studien identifizierte ich die Bindungsoberflächen von CBP und USF-1 füreinander und konnte diese im Falle von USF-1 auf einen Bereich von 22 Aminosäuren eingrenzen. Die Deletion dieser Domäne zieht nicht nur den Verlust der Transaktivierungs-fähigkeit eines HIV-1 LTR Reportergens durch USF-1 nach sich sondern unterbindet auch den Synergismus mit Ets-1.

Zusammenfassend bieten diese Ergebnisse einen detaillierten Einblick in die molekularen Mechanismen, die der Kooperativität des Ets-1/USF-1 Komplexes zugrunde liegen. Sie deuten darauf hin, dass die Interaktion zwischen den Transkriptionsfaktoren zu erheblichen Konformationsänderungen führt die sowohl die DNA-Bindung des Komplexes als auch dessen Eigenschaften bei der Transaktivierung beeinflussen. Diese Erkenntnisse über das vielseitige Wechselspiel zwischen Ets-1 und USF-1 legen ein Model nahe, bei dem Transkriptionsfaktoren nicht als individuelle Einheiten zu sehen sind sondern als Bestandteile größerer Proteinkomplexe, die ihre vollständige Funktionalität erst in Verbindung mit anderen Partnermolekülen entwickeln.

To Lowis and Corinna

and to

my parents

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What lies ahead in the future?

"I don't have a mystical crystal ball, but I do have an old cobalt blue aspirin bottle (dated "1899") that was pulled out of an old garage dump in Colorado. This blue bottle is not exactly Aladdin's lamp, but it does reveal visions of the future….
What should we do?
First, there is every reason to conclude that we should continue doing what we have been doing. That is, we should continue to develop in vitro systems to decipher the molecular mechanisms by which sequence-specific factors and cofactors regulate transcription. We should devise novel assays for the discovery and isolation of new activities…"

James T. Kadonaga (Cell 2004)

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1. Introduction

Until the early sixties cells were believed to loose continuously genes during differentiation to give rise to the various cell types of a multicellular organism. This hypothesis was disproved when J. Gurdon (Gurdon, 1962) has discovered that nuclei from intestinal epithelium tadpole cells implanted into enucleated frog egg cells can develop into normal tadpoles. This finding was the first indication for the nowadays well accepted principle that in general all cells contain the same genetic information. More recently, a spectacular publication reported the cloning of a sheep by the same principle (Wilmut et al., 1997) proving that the findings in amphibians are valid in mammals, too. A few weeks ago South Korean scientists even generated cloned human blastocysts (Hwang et al., 2004) with nuclei from differentiated ovarian cells.

What is it than that makes one cell type different from another?

The identity and function of each cell is determined by the proteins it is made up of. Proteins are generated by the tightly controlled process of *gene expression.* This term commonly refers to the entire process of gene transcription into RNA, its processing and transport, the translation of the RNA into the protein and posttranslational mechanisms regulating protein stability. It remains an important question how a cell determines which complement of protein needs to be expressed to make up its particular cellular identity.

Each cell type uses a different subset of the genomic information to acquire their specific properties or to respond to a changing environment. To be able to adapt to changes in the environment cells must be able to sense differences.

The initial findings about the control of gene expression have been made in bacteria since in prokaryotes gene control serves mainly to allow a single cell to adjust to changes in its nutritional environment so that its growth and division can be optimized. Jakob and Monod found in the early 60's that a group of genes coding for enzymes essential for the use of Galactose as a nutritional source become induced by the presence of Lactose. The transcriptional control is exerted by a protein tetramer consisting of four LacI molecules that assemble to the Lac repressor. The Lac repressor binds to a DNA stretch, the Lac operon, that is situated 5' of the transcriptional start site and which overlaps with the promoter thus avoiding the

assembly of the general transcription machinery. Binding of Lactose to the repressor induces a conformational change leading to the liberation of the promoter and subsequent production of the enzymes required for Lactose metabolism. This very simple mechanism of gene regulation set the basis for decades of research in which many variations of transcriptional regulation became elucidated.

The evolutionary step from single to multicellular organisms has put new demands on gene regulation, which now has to assure the control of cellular identity, the cellular position in the body plan and intercellular communication reflected in gene regulation programs that underlie embryologic development, cellular differentiation and coordination of multicellular efforts such as the immune response.

To respond to these exigencies transcriptional control has reached a much higher level of complexity in metazoans. Since the number of genes is in the same order of magnitude in invertebrates and humans increased organismal complexity must also be reached on the level of regulation. With this in mind we had to recognize that a single transcription factor can not be seen as a switch that decides if a gene becomes shut on or off. Instead it became more and more clear that a huge number of different transcription factors and coregulatory proteins control any given gene and that expression of a gene depends on the interplay of positive or negative activities of these factors.

Also viruses depend on the cell's general transcription factors to assemble initiation complexes within their basal promoters and rely on cellular transcription factors and coactivators that stimulate and regulate transcription (see below). Whereas simple viruses like some retroviruses totally depend on control by cellular transcription factors. More complex viruses such as DNA viruses or the human immunodeficiency virus also encode their own regulatory proteins that interact with and modulate the activities of cellular factors. These viral proteins serve to activate or further stimulate the transcription of viral as well as cellular genes, and they act at multiple steps in the transcription process.

The human immunodeficiency virus has been extensively studied with respect to its transcriptional regulation and findings have been very instructive for general concepts of gene regulation.

1.1. Transcriptional regulation

According to recent estimates, a human cell has between 30,000 and 40,000 coding genes, but only a fraction of these are expressed in any cell lineage. Gene expression profiles of a given cell type undergo dramatic changes throughout development, differentiation, and the cell cycle, presenting the gene regulatory apparatus of a cell with a phenomenal degree of complexity. Cells deal efficiently with this enormous task by using transcriptional regulators in a combinatorial way. Thus, unique combinations of transcription factors convey specificity of gene expression and allow the use of each transcription factor at multiple gene loci. One advantage of this strategy is that even widely expressed transcription factors or cofactors can contribute to tissue-specific gene expression.

The cell disposes of several mechanisms to control the expression and the amount of active proteins (Figure 1): Accessibility of genes in the context of chromatin, transcriptional control, control of RNA processing as well as its transport and stability, translational control and finally the control of the activity of the resulting proteins. For many genes control of transcription is the most important since it represents the first step of expression. By this strategy cells avoid at the beginning the wasteful investment in energy consuming processes for protein production.

Figure 1

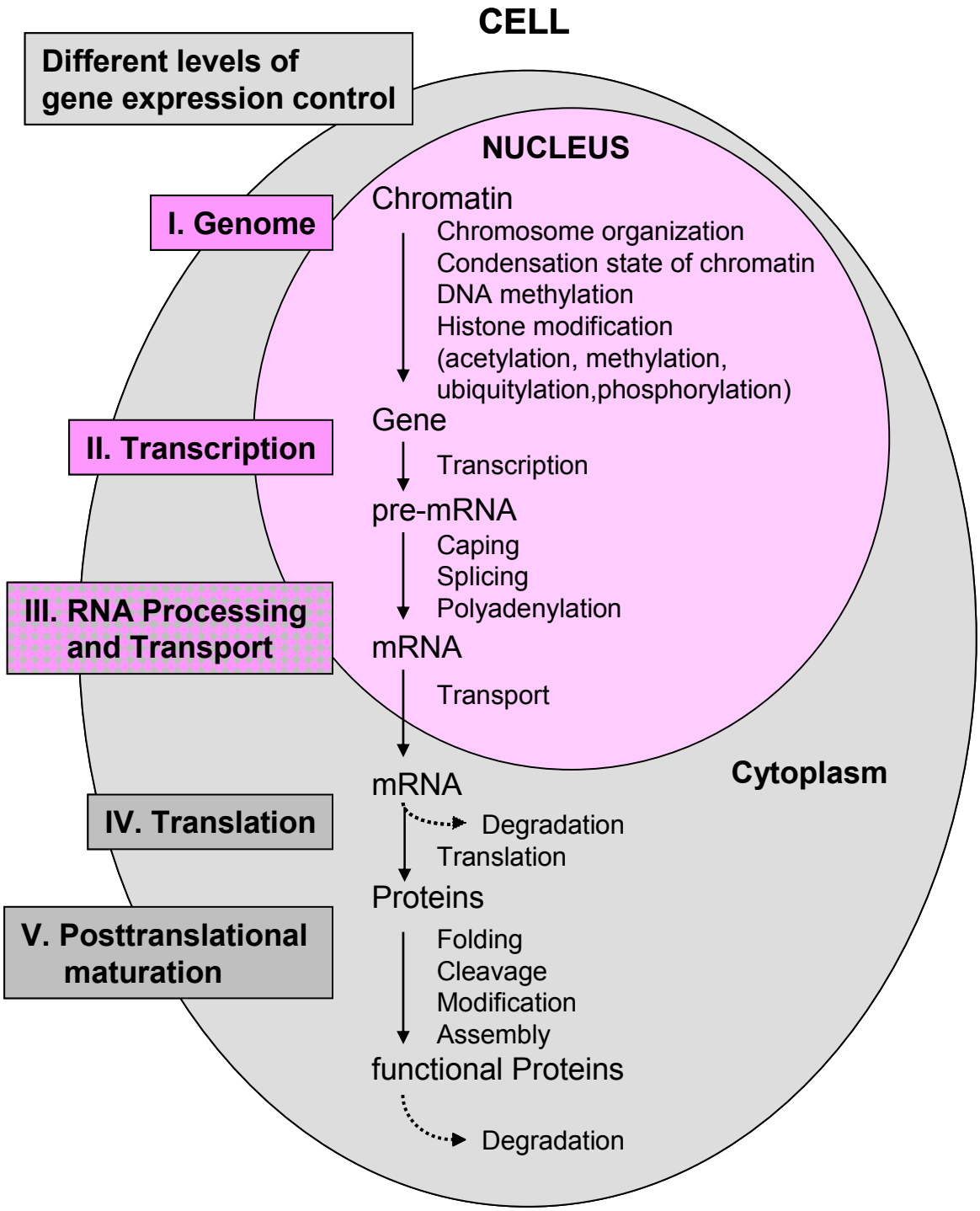


Fig.1 Schematic model of the different levels and steps of gene expression control.

1.1.1. Basic elements for transcriptional control

Two basic elements are required for transcriptional regulation:

(1) Recognition elements on the DNA for the basic transcription machinery and transcription factors which are situated in the promoter, in upstream promoter elements and enhancer/silencer regions and

(2) the protein family of transcription factors.

The promoter contains basic recognition sites as the TATA box or the initiator element (Inr) which are recognized by proteins of the basic transcription machinery. The TATA box is first bound by the TATA-binding protein (TBP) which then subsequently recruits several TBP-associated factors (TAFs) to form TFIID. This complex is completed by further binding of the general transcription factors (TFIIA, TFIIB, TFIIF, TFIIE, TFIIH and TFIIJ) and RNA Polymerase II to form the functional Polymerase II Transcription-Initiation Complex (pol II machinery). Inr binding proteins also allow the assembly of the basic transcription complex including TBP that in TATA less promoters is not directly binding DNA but is recruited by protein-protein interactions.

The upstream promoter element was initially believed to contain binding sites for transcription factors that are required for the constitutive expression of genes. In contrast enhancer and silencer regions were defined by their capacity to act over wide distance up- and downstream of the transcription start site and to be bound by tissue specific transcription factors. Through the analysis of transcription control regions of hundreds of genes, however, this distinction has softened since enhancer regions can lie in close proximity to the promoter of some genes and the upstream promoter element can equally contain binding sites for tissue- or signal specific transcription factors.

Typical transcription factors are modular. This has been shown the first time by the generation of fusion proteins between the DNA binding domain of LexA and Gal4 that activate transcription in a LexA binding-site-dependent manner showing that Gal4 transactivation and DNA binding function can be separated and act independently of each other (Brent and Ptashne, 1985). Later, modules with different functions have been identified that confer for example dimerization, activation/repression, responsiveness to ligands, autoinhibition and interaction with other proteins.

Transcription factors contain a variety of structural motifs that are able to recognize specific DNA sequences. They have been subdivided into transcription factor families that share structurally similar DNA binding domains (Mitchell and Tjian, 1989). Among those are winged helix-turn-helix proteins like for example ETS proteins and basic helix-loop-helix leucine zipper proteins as the USF proteins.

1.1.2. General concept of transcriptional control

In the last decades many modes of transcriptional control had been identified and the concepts of transcriptional regulation evolved.

(1) A classical transcription factor is defined as a protein that can bind to DNA and exert a positive or negative effect on the transcription of a given gene via its transactivation or repression domain, respectively. Nowadays, the definition of a transcription factor becomes broader with the discovery of transcription factor complexes that are composed of DNA binding proteins interacting with cofactors that can exert or modulate transcriptional activity without binding DNA themselves.

(2) Gene expression is also determined by the chromatin context. Genes lying in tightly packed regions can not be transcribed because most transcription factors have no access to their binding sites. Several mechanisms have been shown to be required for “epigenetic” changes that allow the reversible alteration of transcriptionally active to permissive, repressive or permanently silent chromatin states (Fisher, 2002). Among those are DNA methylation (Bird and Wolffe, 1999), covalent modification of histone tails (Khorasanizadeh, 2004) and the spatial restriction of loci to nuclear domains. For the specific targeting of genes for these types of alteration it is assumed that transcription factors can recruit protein partners that are able to further modify chromatin or to localize a region in specific domains (Gasser, 2001).

(3) Transcription factors themselves also underlie control. Some transcription factors, as nuclear receptors for example, contain a regulatory module that confers activity upon ligand binding. Others are retained in the cytoplasm until a specific trigger allows them to enter into the nucleus to function in gene regulation. Posttranslational modification is another mechanism to control the activity of transcription factors. Among those are phosphorylation, acetylation, ubiquitylation and sumoylation. These modifications affect transactivation and DNA binding activity, protein stability and localization or can promote specific protein-protein interactions.

(4) As mentioned above, some regulatory regions (enhancer/silencer) recognized by transcription factors act over a wide distance. To bring these enhancer/silencer bound factors in close proximity to the pol II machinery some transcription factors are able to bend DNA thus creating loops that enable these distant regions to come together. Examples for this class of architectural factors are the HMG (Kim and Maniatis, 1997) and LEF families (Eastman and Grosschedl, 1999).

1.1.3. Specificity in transcriptional regulation

A difficult problem in biology is the issue of specificity of action by molecules that in sequence and structure are almost identical. This problem is well illustrated by families of evolutionarily conserved DNA binding proteins -particularly transcription factors- that need to activate and repress unique sets of target genes.

A common pathway to specificity is the formation of protein partnerships in which two or more proteins that bind DNA together, display added affinity and expanded sequence requirements. Achievement of specificity, however, is not only determined by more selective and enhanced DNA-binding properties of transcription factor complexes but also by further assembly of higher ordered structures between complexes bound to promoter and enhancer regions, coregulatory proteins and the basic transcription machinery. Detailed studies about T cell receptor alpha (TCRα) and Interferon beta (IFNβ) gene activation lead to the concept of “enhanceosome” formation (Carey, 1998; Giese et al., 1995; Kim and Maniatis, 1997).

Enhanceosome assembly is dependent on the arrangement of activator recognition sites and the precise complement of bound activators, which together generate a network of protein-protein and protein-DNA interactions unique to a given enhancer. Formation of enhanceosomes integrates two levels of specificity. Cooperative DNA binding by different transcription factor complexes and the formation of a concerted activation surface that is complementary to “target” surfaces on coactivators and the basal transcription machinery. This process is facilitated by architectural proteins that allow protein-protein interactions over distance by bending the DNA (Grosschedl, 1995). The enhanceosome then facilitates the formation of the basal machinery and reciprocally the basal machinery stabilizes enhanceosome assembly (Figure 2).

Figure 2

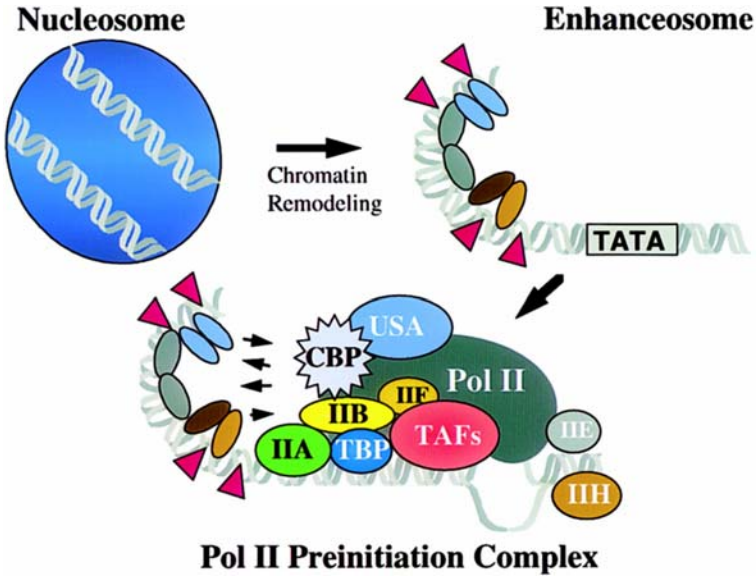


Fig. 2 Concept of enhanceosome and preinitiation complex assembly. The complex assembles in a multistage process. In the beginning, several activators bind to chromatin to open up the gene locus. Thereafter, multiple sequence-specific activators (ovals) and DNA-bending proteins (triangles) engage in cooperative protein-protein interactions to form a stable enhanceosome. By the assembly the transcription factors form a composite interaction surface that serves to recruit transcriptional coactivators that results in cooperative recruitment of the pol II machinery and synergistic transcription. The reverse and forward arrows indicate reciprocity in the interactions. Although the process is delineated into separate steps, the reciprocity may drive concerted assembly of a transcriptosome. Figure taken from Carey (Carey, Cell 98).

The network of ETS- domain transcription factors represents a well studied model for how combinatorial gene expression is achieved. These transcription factors have been described to interact with a multitude of coregulatory partners to elicit gene-specific responses and drive distinct biological processes.

1.2. Ets proteins

The first and name giving member of the family, Ets-1, was discovered in the early 1980´s as part of an oncogenic fusion with the product of the *c-myb* proto-oncogene in the E26 avian leukemia virus. Up to now more than 45 members of this family have been characterized as transcriptional activators and inhibitors in eukaryotes. The *ets* genes encode regulatory transcription factors that share a highly conserved DNA-binding domain consisting of ~85 residues - the ETS domain. The

proteins show significant diversity outside of the ETS domain and the similarities between families members in these domains have been used to classify them into 9 subfamilies (Figure 3). The ETS transcription factors play roles in important biological processes, including cellular proliferation, differentiation, development, transformation, immune response and apoptosis (Ghysdael and Boureux, 97; (Graves and Petersen, 1998). ETS proteins became a model system to study the molecular mechanisms of transcriptional control, including how transcription factors bind DNA, modulate promoter activity, and respond to signaling input (reviewed in (Li et al., 2000; Oikawa and Yamada, 2003; Sharrocks, 2001; Yordy and Muise-Helmericks, 2000)).

Figure 3

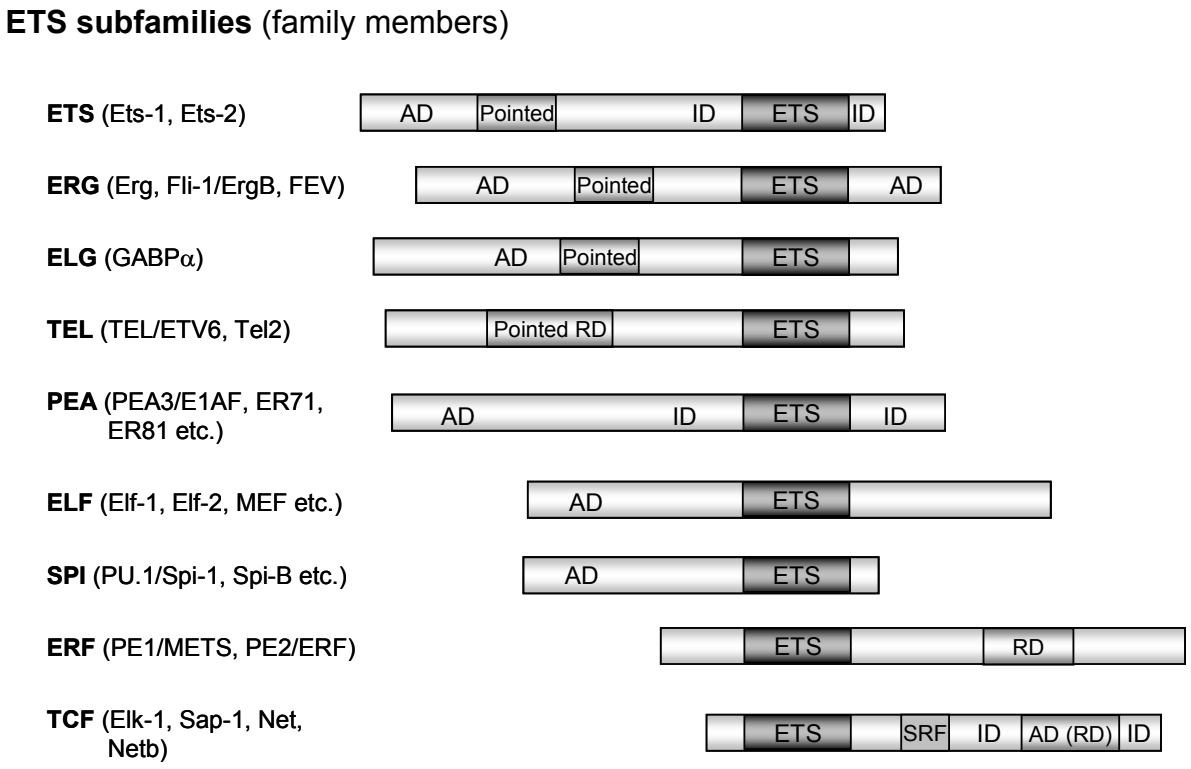


Fig. 3. Schematic presentation of the structure of different ETS family members ETS, Ets DNA-binding domain; HLH, helix–loop–helix domain; Pointed domain; AD, activation domain; ID, auto-inhibitory domain; RD, repression domain.

1.2.1. The ETS domain

Studies on the ETS domains of Ets-1 (Donaldson et al., 1996; Garvie et al., 2002), PU.1 (Kodandapani et al., 1996) and Elk-1 (Mo et al., 2000) showed a high degree of structural conservation, with each containing three α-helices and four β-sheets. The tertiary structure of Ets-1 shows that the three helices within the ETS domain fold into a helix-turn-helix (HTH) element that packs against a four-stranded, anti-parallel beta-sheet (Werner et al., 1995). The beta-sheet with its accompanying loops provides a winged appearance which lead to the grouping of ETS transcription factors into a large structural class of DNA binding proteins, termed winged helix-turn-helix (wHTH) proteins (Brennan, 1993).

The ETS domain is not only establishing protein-DNA contacts but is also a target for either intramolecular protein-protein interactions or for co-regulatory transcription factors. These interactions play an important role in the regulation of the function of ETS transcription factors by controlling DNA binding and providing specificity.

1.2.2. Structural features for ETS protein DNA binding

All ETS-domain proteins bind to sequences that contain a central GGAA/T motif – the Ets protein binding sites (EBS). However, individual family members can select specific nucleotides over an 11-base-pair sequence, which is centered on this motif (Graves and Petersen, 1998). In general ETS proteins bind to DNA as monomers even though a recent publication reports the formation of Ets-1 homodimers on the stromelysin-1 promoter (Baillat et al., 2002). The main protein-DNA contacts are from residues that are located in the third α-helix, in the 'wing' between β-strands 3 and 4 and also a loop between α-helices 2 and 3 (Figure 4) (Donaldson et al., 1996; Werner et al., 1997). Additionally, helix 1 is proposed to play a role in DNA binding by contacting a DNA backbone phosphate. For optimal DNA binding the alignment and positioning of this helix 1 is crucial. Regulation of conformation of this module in the ETS context is an important step in controlling Ets-1 activity (Wang et al., 2002).

Despite some binding preferences of some ETS-domain proteins to a given recognition site, there is a large overlap in binding specificities. Therefore, other mechanisms are required to recruit the right ETS-domain protein to a specific promoter and to avoid promiscuous binding to ETS binding motifs. A crucial

mechanism for ETS-domain proteins to achieve specificity is the interaction with co-regulatory proteins as discussed later.

Figure 4

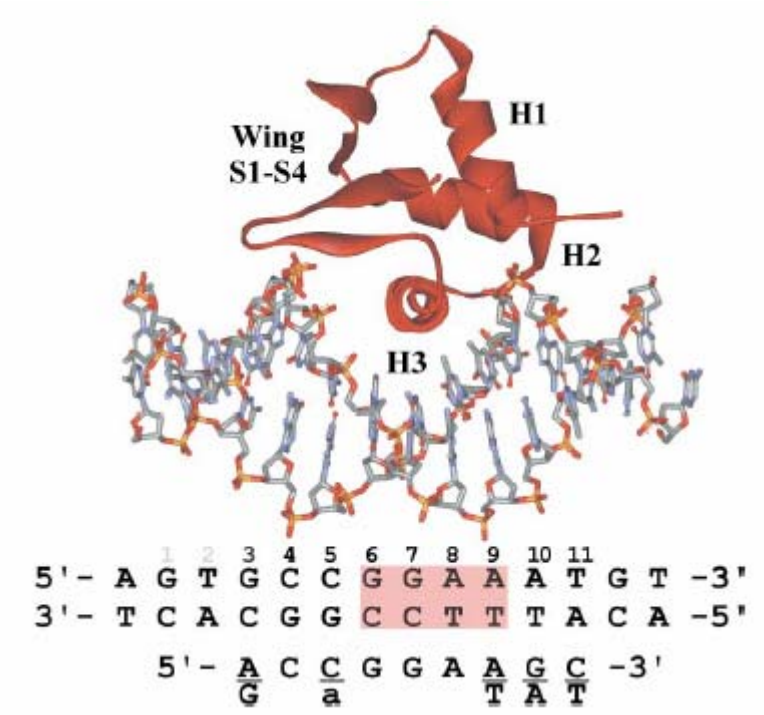


Fig. 4 ETS Domain-DNA Interaction. Amino acids 331–415 of Ets-1 and numbered duplex; positions 3–11 show sequence preference. Highlighted positions 6–9 define the core recognition sequence. Lower sequence is "selected" consensus sequence for Ets-1. (Figure from Pufall, Graves Structure 02)

1.2.3. Biological roles of Ets proteins

The activity of ETS proteins is determined by the respective cellular context and is influenced mainly by two ways: (1) Posttranslational modification, affecting DNA-binding, protein interaction and transactivation. (2) Interaction with coregulatory proteins, i.e. other transcription factors and coactivators that influence promoter specificity, DNA-binding and transactivation potential.

The expression of ETS family proteins is often restricted to specific cell lineages and it has been shown that they are involved in development and differentiation by controlling the enhancer or promoter activities of cell line specific

genes. Moreover, deregulated expression of some ETS family members due to proviral insertion, chromosome translocation and mutation is associated with leukemia and specific types of solid tumors, possibly due to their role in cell proliferation and differentiation. Several ETS family proteins also participate in malignancy of tumor cells including invasion and metastasis by activating the transcription of several protease genes and angiogenesis-related genes.

1.2.4. Selected biological roles of Ets-1

The founding member Ets-1 has been extensively studied and shown to play a role in all processes mentioned above. Some selected functions will be described more in detail.

1.2.4.1. Ets-1 in Growth control

Ets-1 as nuclear target of signaling pathways contributes to adjustments in proliferative or cell cycle behavior of cells. It has been shown, that Ca^{2+}-signalling dependent phosphorylation of the N-terminal autoinhibitory module dramatically reduces DNA-binding (Cowley and Graves, 2000). By contrast, the transactivational function of Ets-1 is activated by Ras-MAP kinase signaling (Yang et al., 1996a). Both signal pathways are used to transmit signals from growth factors and stress.

Activation of Ets-1 via Ras-MAP kinase signaling has been reported to enhance the *junB* promoter (Coffer et al., 1994). JunB is part of a family of bZip proteins that can dimerize in various combinations to form activating protein 1 (AP-1). AP-1 factors constituted of Jun and Fos family members are implicated in the control of proliferation (Shaulian and Karin, 2002). Ets-1 also contributes to the expression of the cell cycle relevant c-Myc, a transcription factor that plays important roles in the induction of cell proliferation and apoptosis (Roussel et al., 1994).

Furthermore, Ets-1 has been reported to be pro-apoptotic as well as anti-apoptotic in some cases. Ets-1 appears to be required for survival and activation of T cells, since *ets-1*$^{-/-}$*Rag-2*$^{-/-}$ chimeric mice display a marked decrease in the number of mature T cells and a severe deficiency in proliferation in response to activating signals with increased rates of spontaneous apoptosis in T cells (Muthusamy et al., 1995). In contrast, a recent report has revealed that Ets-1 is required for the formation of a stable DNA-p53-CBP complex to induce pro-apoptotic genes in the process of UV-induced apoptosis in embryonic stem (ES) cells (Xu et al., 2002).

1.2.4.2. Ets-1 in hematopoietic differentiation

Differentiation of hematopoietic progenitors

Determination of hematopoietic cell differentiation seems to be controlled by coordinate action of extracellular signals and a cascade of appearance of several critical master regulators of transcription factors for specific cell lineages (Graf, 1998; Tenen et al., 1997). Several ETS family proteins are preferentially expressed in certain lineages of hematopoietic cells and also play important roles in their development, commitment and differentiation. Ets-1 is initially expressed in the blood island of the yolk sac where hemangioblasts, the common precursors of vascular and hematopoietic lineages, are present (Maroulakou and Bowe, 2000). This may account for the expression of Ets-1 in both endothelial cells and certain lineages of hematopoietic cells.

Erythrocytes:

Overexpression of Ets-1 promotes erythroid differentiation of K562 cells (Clausen et al., 1997). Furthermore, it is known that Ets-1 transactivates the transferrin receptor gene essential for erythroid differentiation. Overexpression of MafB, an AP-1-like myelomonocyte-specific transcription factor, in an erythroblast cell line down-regulates expression of the endogenous transferrin receptor gene by a direct interaction with Ets-1 and thus inhibits erythroid differentiation (Sieweke et al., 1996).

Lymphocytes:

Ets-1 is expressed throughout lymphocyte differentiation and present at high levels in mature B-cells, T-cells and natural killer cells. However, gene inactivation in mice showed that it is not required for the development of B- and T-cells but appears to be essential for their survival and maturation (Bories et al., 1995; Muthusamy et al., 1995).

Functional interaction of Ets-1 with Stat5 has been shown to contribute to the proliferative response to interleukin-2 (IL-2) in T cells (Rameil et al., 2000). It is notable that the IL-2 induced Ets-1/Stat5 complex forms *in vivo* prior to binding to DNA and that DNA binding depends on Stat5 binding sites. Thus, direct Ets-1 DNA

binding is not required. Ets-1 controls several T cell-specific genes including the T cell receptor α (TCRα) and TCRβ genes in cooperation with CREB, TCF-1, AML1 and GATA-3 (Halle et al., 1997; Ho et al., 1990; Kim et al., 1999).

1.2.4.3. Ets-1 and tumor development

Up-regulation of expression of the *ets-1* gene has been documented in many types of human tumors. Generally, expression levels of Ets-1 correlate well with the grade of invasiveness and metastasis (Behrens et al., 2001) and therefore can be useful for predicting poor prognosis of the cancer patients. Expression of genes encoding for enzymes involved in degradation of the extracellular matrix (ECM), such as MMP-1 (collagenase-1), MMP-3 (stromelysin-1), MMP-7 (matrilysin), and MMP-9 (type IV collagenase/gelatinase B) is regulated by Ets family proteins including Ets-1. Hence, it is strongly suggested that Ets-1 contributes to tumor invasion and progression through activation of these enzymes. Indeed, expression of these ECM remodeling enzymes is detected along with expressing *ets-1* mRNA in tumor cells and/or stroma cells (Davidson et al., 2001).

Several ETS factors are implicated in the differentiation of endothelial cells that later will form blood vessels. This is interesting in the context of tumor development since beyond a certain size growing tumors depend on vascularisation for nutrient supply. The microenvironment of a growing tumor mass is significantly oxygen-deprived and hypoxia induces expression of *ets-1* via the activity of hypoxia-inducible factor-1 (HIF-1) (Oikawa et al., 2001). Thus, chronic hypoxia in rapidly growing tumors results in induction of Ets-1 in tumor cells and/or stroma cells to subsequently induce angiogenesis-related genes.

1.2.5. Ets-1 is autoinhibited for DNA binding

Autoinhibition is a widespread phenomenon that plays a key role in the regulation of proteins. In general an intramolecular interaction interferes directly or allosterically with a function of a “targeted” domain. An advantage of this way of regulating a proteins activity is the high effective local concentration that is generated by tethering of the interacting counterparts. Thus, relatively small surface areas and weak interactions are sufficient for inhibition. Furthermore, the protein can not “escape” the regulation since it is physically linked to the on-site repressor.

The autoinhibitory mechanism that controls the transcription factor Ets-1 has been studied intensively. Autoinhibitory elements in Ets-1 were discovered by the observation that deletions of regions flanking the ETS domain N- or C-terminally, enhanced DNA binding (Graves et al., 1998; Hagman and Grosschedl, 1992; Lim et al., 1992). Structural studies using NMR spectroscopy identified 4 inhibitory helices within these regions, two in the N-terminal region (inhibitory helix HI-1 and HI-2) and two at the C-terminus (Helix4 and Helix5) (Garvie et al., 2002; Skalicky et al., 1996). The N-terminal inhibitory helix, HI-2, makes extensive contacts with the C-terminal inhibitory helices and with helix H1 of the ETS domain on a surface of the protein opposite of the DNA-binding surface. In addition, helix HI-1 packs against inhibitory helices HI-2 and H4 to form a hydrophobic core. In this way, helix HI-1 serves as a critical cross brace connecting the N-terminal and C-terminal inhibitory regions and stabilizing the inhibitory modules (Garvie et al., 2002).

Upon binding to DNA the inhibitory modules undergo a conformational change. The most dramatic event during the transition of autoinhibited Ets-1 to the DNA bound form is an unfolding of the inhibitory Helix HI1 (Petersen et al., 1995). Autoinhibition of Ets-1 is believed to be due to the energetic cost of altering the conformation of the inhibitory modules. This energetic cost of DNA binding could be compensated by interaction with other transcription factors (Figure 5).

However, a key question regarding the mechanism of autoinhibition centers on how inhibitory helices that pack on the non-DNA binding face of the protein can influence the affinity of Ets-1 for DNA. One model that has been proposed is that the packing of all four inhibitory helices against the ETS domain favors a conformation of Ets-1 that cannot form optimal contacts with the DNA. Wang et al. have shown that Helix-1 can directly contact DNA and is interacting with the inhibitory modules thus providing a link between autoinhibition and DNA binding. They have shown that Helix-1 establishes additional contacts to DNA through the exact orientation in the overall ETS structure. However, this position depends on relieve of interaction with the autoinhibitory modules which is achieved by the unfolding of Inhibitory Helix HI-1 (Wang et al., 2002). These data provide an elegant model for the molecular basis of autoinhibition of Ets-1.

Figure 5

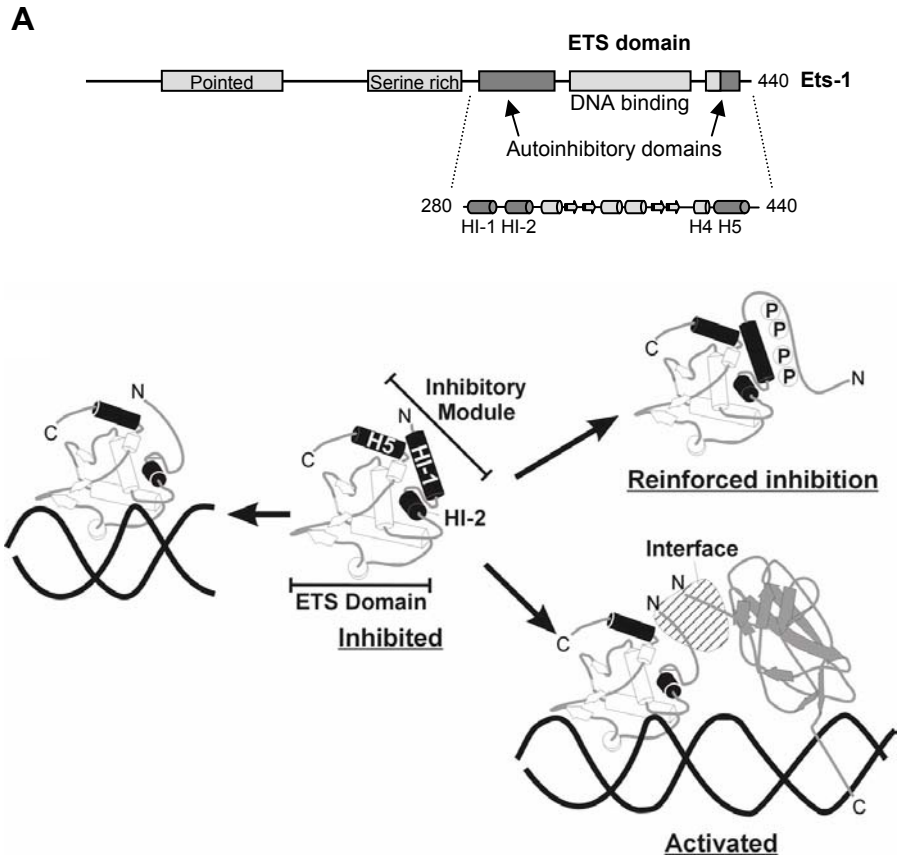


Fig. 5 Autoinhibition of Ets-1 DNA binding. (*A*) Domain structure of eukaryotic transcription factor Ets-1. The pointed domain functions in protein interactions. The ETS domain is flanked by autoinhibitory elements. The serine-rich region is the site of inhibitory phosphorylation. Secondary structure of ETS domain and inhibitory elements. (*B*) Ets-1 autoinhibition and regulation of DNA binding. Model of the autoinhibitory module structure (Garvie JBC 02) (*center*). DNA binding disrupts intramolecular contacts between the ETS domain and the autoinhibitory module, characterized by the unfolding of HI-1 (*left*). Phosphorylation of the serine-rich region in response to calcium release further inhibits DNA binding by stabilizing the autoinhibitory module (*upper right*) (Cowley & Graves 2000). A protein partnership with other transcription factors (here: AML-1) counteracts the inhibitory mechanism by interacting with the autoinhibitory module (Kim EMBO 99) (*lower right*). The DNA-binding domain of AML-1 is adapted from Bravo et al. (2001). Hatched oval represents the N-terminal region necessary for cooperative DNA binding for which there is no structural information. Modified figure from (Pufall, Ann. Rev. Cell Dev. Biol. 02).

1.2.6. Protein interaction of Ets-1 with other factors

Protein-protein interaction has turned out to be an elementary property of probably all transcription factors and has been especially well established for Ets-1. Indeed, transcription factor complex formation by Ets-1 with other factors serves several functions.

(1) As discussed above, the DNA binding capacity of Ets-1 is autoinhibited until an appropriate trigger is in place. DNA binding is influenced either by phosphorylation (Cowley and Graves, 2000) or by interaction with coregulatory transcription factors (Figure 5). Sequences flanking the EBS not only partially define individual ETS family member binding specificity but contain also adjacent binding sites for many transcription factors of diverse families. These composite binding sites are bound by specific Ets-1/partner transcription factor complexes, depending on the cellular context and lead to higher affinity and stability in DNA binding.

The interaction of Ets-1 with AML-1 leads to transcriptional regulation on the T cell receptor β (TCRβ) chain enhancer (Kim et al., 1999) and on the Moloney murine leukemia virus enhancer (Goetz et al., 2000). Both proteins are autoregulated for DNA binding and it has been shown that the physical interaction between the autoinhibitory domains of Ets-1 and AML1 allows cooperative DNA binding (Kim et al., 1999). A complex of Ets-1 with the paired box transcription factor Pax-5 acts on the Ig-alpha encoding *mb-1* gene in B-cells (Fitzsimmons et al., 1996). Structural studies show that interactions between the paired domain in Pax-5 and the DNA binding helix in Ets-1 result in changes in protein-DNA interactions by Ets-1 and hence promote its ability to recognize a suboptimal site (Garvie et al., 2001). The bHLH Zip protein USF-1 has been shown to cooperate with Ets-1 in binding of composite recognition elements on the HIV-1 LTR (Sieweke et al., 1998).

(2) Modulation of Ets-1 transcriptional activity through complex formation. Interaction between Ets-1 and other transcription factors results in either activation or repression of specific target genes. In the complexes discussed above, Ets-1 acts as transcriptional activator. By contrast, Ets-1 interaction with MafB, an AP-1 like protein, inhibits Ets-1 mediated transactivation of the transferrin receptor gene and inhibits erythroid differentiation (Sieweke et al., 1996).

(3) CREB binding protein (CBP) and the highly homologous p300 have been shown to interact with Ets-1 and to induce higher transactivation capacity when overexpressed (Jayaraman et al., 1999; Yang et al., 1998).

Thus, Ets-1 in itself combines all the requirements to take part in all levels of higher ordered enhanceosome assembly as shown in the context of the enhanceosome controlling TCRβ expression (Giese et al., 1995).

1.3. USF

Basic helix-loop-helix (bHLH) proteins are a family of DNA-binding proteins characterized by their ability to recognize specific DNA sequences, termed E-boxes (CANNTG) through their basic region. They are able to form homo- or heterodimers via their HLH domain. E-box elements were first identified in the immunoglobulin heavy-chain (IgH) intronic enhancer and have since been found in a large number of promoter and enhancer elements. These proteins have originally been grouped into three classes based upon structural characteristics and pattern of expression. The class A proteins are ubiquitously expressed and readily bind DNA either as homo- or heterodimers, with some of them implicated in cellular differentiation. Class B proteins are expressed in a tissue-specific manner and form heterodimers with class A bHLH proteins. Some of these factors such as MyoF, myogenin and *myf-5* are implicated in muscle development (Lassar et al., 1994). The last group, class C, forms homo-and heterodimers with class C but not with class A or B bHLH proteins. Among class C are factors such as Myc, Max and Mad that are implicated in cellular proliferation, differentiation and apoptosis (Murre et al., 1989). Upstream stimulatory factor (USF) is another member of this class that is broadly expressed in mammals and shall be described more in detail (Figure 6).

USF was initially characterized as a transcription factor implicated in the regulation of the adenovirus major late promoter (MLP) (Sawadogo and Roeder, 1985). USF proteins belong to the class C of bHLH proteins and contain in addition a leucine zipper (Zip) motif that confers further dimerization capacity (Gregor et al., 1990). Two distinct USF genes, referred to as USF-1 and USF-2, have been characterized and shown to encode full-length USF-1 and USF-2 proteins of 43 and 44 kDa, respectively (Sirito et al., 1994). The C-terminal bHLH Zip domains of USF-1 and USF-2 are highly conserved, and direct the formation of USF homo- and heterodimers that interact with DNA with similar specificities (Sirito et al., 1992). The major form of USF present in most cell lines is the USF-1/USF-2 heterodimer. USF-1 homodimers are less abundant and USF-2 homodimers are usually very rare (Sirito et al., 1992). The cognate DNA binding element for USF proteins, the E-box, contains the core nucleotide sequence CANNTG. Another very well conserved domain between USF1 and USF2 is a small domain termed USF-specific region (USR) that is located immediately upstream of the basic region. In contrast, the N-terminal regions

of USF1 and USF2 are much more divergent, but were shown to contain additional trans-activation domains (Kirschbaum et al., 1992; Luo and Sawadogo, 1996).

Figure 6

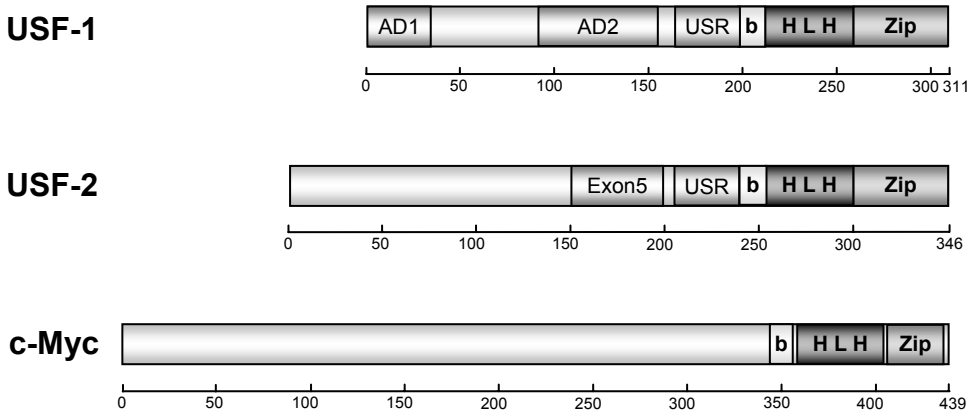


Fig.6 Schematic presentation of the domain structure of human upstream stimulatory factor (USF-1 and USF-2). USF shares highly homologous DNA binding and dimerization domains (bHLH Zip) with other members of the same transcription factor family, as shown here in comparison to c-Myc.The N-terminal region in contrast is very divergent. In USF-1 two independent transactivation domains (AD) have been identified *in vitro* (Bernhard MCB 92). USF-2 also contains two AD which are encompassed by a domain
encoded by Exon5 and a region called USF specific region (USR) that is highly conserved in USF-1 (Luo MCB 96).

1.3.1. DNA binding by USF

Cocrystallization of the C-terminal DNA-binding domain of human USF with its specific DNA-binding site revealed that USF dimers bind DNA as a four-helix bundle, with the basic domain from each monomer contacting half of the DNA-binding site. USF contacts the DNA through a basic region which free in solution has a random structure, but becomes alpha-helically folded upon binding to DNA (Ferre-D'Amare et al., 1994). Furthermore, Ferre-D'amare et al. could provide evidence that USF can form bivalent tetramers with the capacity to bind simultaneously two independent pieces of DNA. This feature is very interesting in the light of a study of Du et al. in which they show that USF can bind to initiator elements as well as to a more upstream E-box in a number of different gene promoters and that deletion of Inr decreases transactivation (Du et al., 1993). It has been proposed that USF could

bend DNA by tetramerization thus bringing other DNA bound transcription factors in close proximity to the basal transcription machinery.

1.3.2. Transactivation domains in USF

In USF-1 as well as in USF-2 two distinct transactivation domains (TA) have been characterized (Kirschbaum et al., 1992; Luo and Sawadogo, 1996) respectively). Most of the N-terminal parts of both transcription factors share little homology. In USF-1 TAs are composed of residues 1-40 and 100-130 (Kirschbaum et al., 1992). TAs in USF-2 are defined as Exon 5 (aa158-199) and a region called USF specific region (USR, aa208-230). As mentioned above, the USR domain is highly conserved in USF proteins and is involved in transcriptional activation. This proposes also an activating role of USR in USF-1. It appears that the USR region plays an important role in transactivation of promoters containing an initiator element (Luo and Sawadogo, 1996).

1.3.3. Biological role of USF

Both forms of the upstream stimulatory factor 1 (USF-1) and USF-2 proteins are broadly expressed and therefore it is not surprising that they have been shown to act on a variety of cellular and viral promoters.

To determine biological functions of the two USF family members, gene defective mice have been generated (Sirito et al., 1998). Whereas the knock-out of either of the two USF genes in mice resulted in minor phenotypes as some behavioral abnormalities in *Usf-1*$^{-/-}$ mice and a growth defect in *Usf-2*$^{-/-}$ a double knock-out is embryonic lethal. These data indicate not only that USF plays a major role in embryonic development or general function required throughout development such as proliferation and cell cycle control but also that there is a redundancy between USF-1 and USF-2. However, USF-1 and USF-2 seem to play also unique roles in the regulation of some genes since the phenotypes of the single mutants are different.

Other members of the bHLH Zip family as Myc, Mad, Max, Mitf and TFE (1-3) also recognize E-boxes with a similar affinity which makes it difficult to determine which family members are implicated in the regulation of particular genes. The problem arises how the presence of E-boxes in gene promoters/enhancers can result

in binding of specific bHLH Zip factors. Therefore, I will present some examples in the next paragraphs to show how USF-1 achieves specificity through interaction with transcription factors of the same structural class or others like ETS factors. In the following chapters the term USF will be used when both forms are implicated in transcriptional control as heterodimers and it will be specified if this function can be attributed to one specific USF only.

1.3.4. Selected biological roles of USF

1.3.4.1. Immunology

Transactivation of major histocompatibility complex (MHC) Class II either constitutively or by induction with IFNγ depends mainly on the transcription factor CIITA. Expression of CIITA has been shown to be controlled in several cell lines by the cooperative action of Stat1, a downstream target of the IFNγ pathway and USF-1 which stabilizes DNA binding of the complex (Muhlethaler-Mottet et al., 1998).

Also tissue-specific expression of class MHC Class I genes seems to be controlled by USF (Howcroft et al., 1999). USF mediated MHC Class I expression can be abolished of a splicing variant of USF-2 (U2DeltaE4) that forms heterodimers with USF and prevent transactivation by acting as a dominant negative USF.

A similar mechanism has been proposed for the regulation of the CD2 promoter. This promoter is typical for a group of T cell-specific promoters that lack a TATA box and use multiple sites for initiation of transcription. An "E box" motif located just upstream from the most 5' initiation start site contributes a major effect to the level of basal transcription through binding of USF (Outram and Owen, 1994). In a recent work Rodriguez et al. (Rodriguez et al., 2003) identified Cha as a new member of the bHLHZip family that binds to USF-1 and inhibits USF mediated CD2 expression in resting T cells. After mitogenic activation by PMA/ionophore Cha is rapidly degraded and USF-1 can activate CD2 expression.

1.3.4.2. Hematopoiesis

USF-1 plays a major role in the hematopoietic expression of HOXB4 that subsequently regulates the balance between hematopoietic stem cell renewal and differentiation (Giannola et al., 2000). This effect is dependent on the presence of several growth factors like trombopoietin (TPO), Flt-3 ligand and stem cell factor

(SCF). Signaling by TPO also requires p38 kinase and it has been proposed that the effect of USF-1 for the maintenance of renewal capacity of hematopoietic stem cells is influenced by this pathway (Kirito et al., 2003).

1.3.4.3. HIV-1 activation

USF-1 forms also transcription factor complexes with members of the ETS protein family. In a yeast one hybrid screen USF-1 was identified as interaction partner of Ets-1. Further analysis revealed that both factors cooperate in DNA binding and in transactivation of a HIV-1 LTR reporter construct (Sieweke et al., 1998). Similar results were obtained for PEA3, another ETS family member (Greenall et al., 2001). This aspect of the biological function of USF-1 will be discussed below in more detail.

1.3.5. USF-1 mediated tissue specific or stress induced gene activity

In a recent work Galibert et al. identified USF-1 as an *in vivo* target for the p38 stress activated kinase. They could show that the bHLH Zip factor microphthalmia-associated transcription factor (Mitf) is responsible for the basal expression of *Tyrosinase* a gene involved in the skin tanning process. However, when cells are subjected to stress by UV-irradiation USF-1 becomes phosphorylated by p38 and subsequently *Tyrosinase* expression is highly upregulated (Galibert et al., 2001). The p38 kinase family plays a crucial role during development, differentiation (Nebreda and Porras, 2000) and in the cellular response to stress or pro-inflammatory cytokines. Thus, activation of ubiquitously expressed USF-1 by p38 could provide an explanation how USF acts as a key link between stress signaling and the transcriptional response of a variety of cellular and viral promoters. Moreover, this could explain also how USF that was initially believed to regulate house-keeping genes because of its broad expression pattern takes part in the control of highly regulated genes implicated in development and proliferation. It appears that the broadly expressed USF represents an inactive pool of transcription factor that participates in the early regulation steps of specific target genes when activated by signaling events. For example, USF has been implicated in activated, but not basal transcription of the mouse metallothionein promoter in response to cadmium (Li et al., 1998). Moreover, activation by cytokines of the HIV LTR that contains a USF-binding

site (Sieweke et al., 1998; Zeichner et al., 1991) requires a p38 kinase (Kumar et al., 1996).

1.4. CBP/p300

The related transcription coactivators, CBP [cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein] and p300, are expressed widely and interact with a surprisingly large number of transcription factors via dedicated domains. Thus they could be viewed as general transcriptional regulators. Despite their similarity, loss of function and aberrant regulation or expression of CBP and p300 are associated with distinct phenotypes and diseases in humans and experimental organisms, indicating that certain genes/tissues are more sensitive to changes in CBP and p300 activity than others.

Figure 7

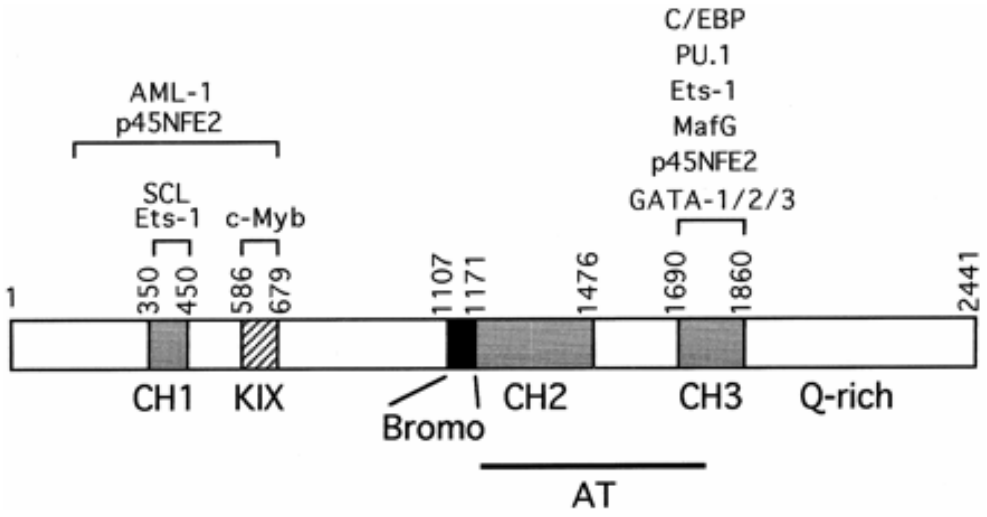


Fig.7 Domain structure of CBP. Hematopoietic transcription factors that bind CBP are indicated. Numbers represent approximate domain boundaries. CH1, CH2, and CH3, CH-rich domains; KIX, CREB-binding domain; Bromo, bromodomain; AT, acetyltransferase domain; Q-rich, glutamine-rich domain.

CBP and p300 share several conserved regions: (1) the bromodomain, which is frequently found in mammalian Histone Acetyl Transferases (HATs); (2) three cysteine-histidine rich (CH)-rich domains (CH1, CH2 and CH3) and the (3) KIX domain. The latter are likely to be important interaction surfaces for other proteins and a number of cellular and viral proteins bind to these regions (Figure 7). CBP/p300 function as transcriptional coactivators and are involved in multiple, signal-dependent transcription events that can influence different physiological processes, including cell growth, proliferation, differentiation and apoptosis (reviewed by (Janknecht and Hunter, 1996). Therefore, it is not surprising that they constitute a target for viral transforming proteins such as adenoviral E1A or the SV40 large T antigen. Formation of viral oncoprotein complexes with CBP/p300 inhibit CBP/p300 and causes loss of cell growth control, enhances DNA synthesis and blocks cellular differentiation (reviewed in (Goodman and Smolik, 2000).

1.4.1. CBP/p300 as an transcriptional integrator

Since the discovery of CBP/p300 as coregulatory proteins many transcription factors have been described to interact with CBP/p300 and multiple mechanisms have been proposed to explain CBP and p300 functions. Although the repertoire of CBP/p300 activities is broad, and their roles must be evaluated in the context of a given gene, certain general and recurring functions can be discussed.

1.4.1.1. CBP/p300 as bridging factors

It has been suggested that CBP/p300 link transcription factors with components of the basal transcription machinery, thereby establishing and/or maintaining transcription-preinitiation complex formation. Thus CBP/p300 can be seen as bridges between DNA-binding transcription factors and the basal transcriptional machinery. This model is supported by the fact that CBP/p300 have been shown to interact with a variety of transcription factors and with components of the basal transcriptional machinery, including TFIIB, TBP, and RNA polymerase II (Goodman and Smolik, 2000).

1.4.1.2. CBP/p300 as Acetyl transferases

The discovery that CBP and p300 are enzymes that catalyze the acetylation of all of the four histone subunits (Bannister and Kouzarides, 1996; Ogryzko et al.,

1996) suggests that CBP and p300 might act in part through regulating chromatin structure. In addition, they associate with other acetyltransferases, including PCAF (Yang et al., 1996b) and GCN5 (Xu et al., 1998) thus leading to the formation of large acetyltransferase complexes with broad substrate specificity. Recruitment of acetyltransferases by transcription factors leads to a local increase in histone acetylation and promotes the opening up of chromatin.

Transcription factors itself also have been shown to be acetylated by CBP/p300. In general, acetylation of transcription factors can alter their activities at various levels, including DNA binding, transcriptional activity, interactions with other proteins, nuclear transport, and protein turnover. Two examples (Tat and NFκB) will be described below.

1.4.1.3. Regulation of CBP/p300

Several recent studies suggest that the enzymatic activities of CBP/p300 can be modulated by protein contacts. For example the viral oncoprotein E1A can inhibit CBP/p300 acetyltransferase activities (Hamamori et al., 1999). Also cellular proteins have been identified that influence CBP/p300 activity by similar mechanisms. For example, PU.1 is a potent inhibitor of CBP/p300 acetyltransferase activity *in vitro* and *in vivo* that can transform erythroid precursor cells and inhibit their differentiation (Hong et al., 2002). Conversely, it can be speculated that CBP/p300 activities might be stimulated by interactions with transcription factors that promote cellular differentiation. Consistent with this possibility, NF-E2 has been shown to augment CBP acetyltransferase activity in vitro when assayed on nucleosomal histones (Chen et al., 2001).

Further regulation of CBP/p300 activities occurs via post-translational modifications, as phosphorylation by cyclin-dependent kinases and mitogen-activated protein kinases that stimulate acetyl transferase activity (Ait-Si-Ali et al., 1998).

1.4.1.4. CBP/p300 as mediator of transcriptional synergy

The model of enhanceosome formation describes that a cooperative assembly of transcription factors leads to a common interface made up by multiple activation domains that recruit CBP/p300. The activational property of CBP/p300 thus could explain the transcriptional synergy often observed between transcription factors.

Conversely, inhibition between nuclear factors has been suggested to result from direct competition for the same binding site in CBP/p300. This mechanism has been invoked to explain the antagonistic effects between GATA-1 and c-Myb during erythroid gene expression (Takahashi et al., 2000).

In summary, CBP/p300 coordinate transcriptional regulation by mediating communication between transcription factors, by regulating transcription-factor activity and by translating cellular signals into a transcriptional response.

1.4.2. Biological function of CBP/p300

Several loss of function studies have provided insight into the biological functions of CBP/p300 in vivo. Alterations in CBP/p300 genes have been observed in various human tumors (Gayther et al., 2000; Giles et al., 1998) and mice defective for both CBP and p300 have been generated. The CBP-/-, p300-/- and double heterozygous mice show similar, embryonic lethal phenotypes. Furthermore, some CBP and p300 heterozygous mice show early lethality indicating that CBP/p300 gene dosage is probably important during development (Yao et al., 1998).

Although p300 and CBP share extensive homology, genetic and molecular analyses suggest that they perform not only overlapping but also unique functions. CBP+/- mice show highly penetrant multilineage defects in hematopoietic differentiation whereas p300 does not (Kung et al., 2000). By contrast a more recent study by Kasper and Brindle (Kasper et al., 2002) revealed that mice homozygous for point mutations in the KIX domain of p300 but not CBP cause multilineage defects in hematopoiesis, including anemia, B-cell deficiency, thymic hypoplasia, megakaryocytosis and thrombocytosis.

1.4.3. Selected examples of CBP/p300 action

The synergy of Ets proteins with CBP and p300-regulated factors led to the hypothesis that they too are regulated by CBP. Indeed, Yang et al. (Yang et al., 1998) showed that the Myb- and Ets-dependent promoter of the myeloid gene CD13/APN is sensitive to the expression of E1A but not mutant E1A defective for CBP and p300 binding. Ets-1 activity is stimulated by coexpressed CBP, and Ets-1 associates with CBP in nuclear extracts. In vitro, the N-terminus of Ets-1 can interact physically with CBP involving the CH1 and CH3 domains of CBP. In support of the functional

importance of the physical interaction between Ets-1 and CBP, the authors demonstrated a good correlation between binding of Ets-1 to the CH1 region and its ability to transactivate.

Another Ets family transcription factor, PU.1, was found to interact with CBP through the activation domain of PU.1 in a yeast 2-hybrid assay (Yamamoto et al., 1999). In this work CBP stimulates PU.1 transcriptional activity of a reporter with multimerized binding sites for PU.1 in transient transfection assays. By contrast, aberrant expression of PU.1 in murine erythroleukemia (MEL) cells inhibits acetylation of transcription factors driving erythroid differentiation like GATA-1 by interfering with CBP acetyl transferase activity (Hong et al., 2002).

Thus, CBP and very likely p300 target a range of transcription factors expressed during hematopoiesis.

1.5. HIV-1 replication

The “aquired immune deficiency syndrome” (AIDS) is caused by infection of CD4 positive cells such as macrophages, T_H-cells and dendritic cells by the “human immunodeficiency virus-1” (HIV-1). After fusion-mediated entry within host cells, uncoating, reverse transcription of the RNA genome and nuclear entry of the preintegration complex, the viral DNA is integrated into the host cell genome, where it is defined as a provirus. The viral RNA genome as well as mRNA for viral proteins is transcribed of the DNA-provirus. The provirus is flanked at the 5’ and 3’ end by “long terminal repeats” (LTRs), sequences crucial for reverse transcription and integration into the host genome.

Viral replication requires transcriptional activation. It has been shown that the 5’ LTR is divided into functional regions designated by transactivation response element (TAR), core, enhancer, and modulatory elements that contain binding sites for several cellular and viral transcription factors (for review, see (Pereira et al., 2000).

The TAR region binds the viral transactivator Tat and the core region contains the initiator (Inr), the TATA box, and three Sp1-binding sites. While the proximal enhancer element binds nuclear factor (NF)-κB and NF of activated T cells (NF-AT) transcription factors, the more distal region harbors numerous target sequences for a variety of cellular transcription factors such as C/EBP, cyclic AMP (cAMP) response

element-binding protein (CREB), Ets-1, USF-1, and nuclear hormone receptors (Figure 8).

Figure 8

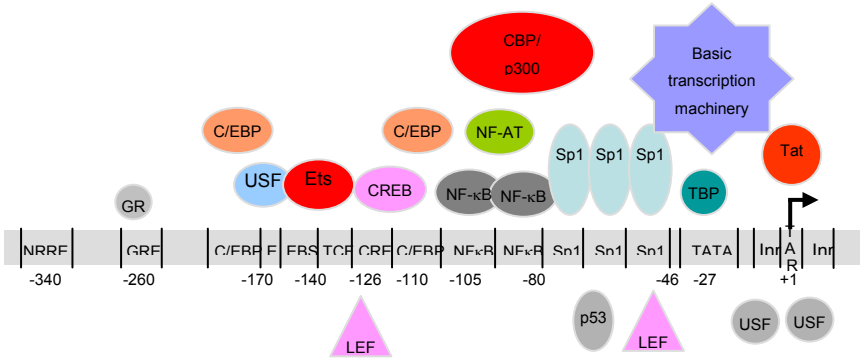


Fig.8 Interactions of endogenous cellular transcription factors with the HIV-1 LTR. The major binding sites within the –356 to 27 region of the LTR are located.

When the HIV-1 provirus is integrated into the host genome, early-phase transcription is regulated by cellular transcription factors and results in the production of early viral gene products. The late phase of transcription is under the control of Tat, which potently enhances gene expression by a direct binding to TAR–RNA and association with cyclin T1 (CycT1), which recruits the cyclin-dependent kinase 9 (cdk9). Formation of this positive transcription-elongation factor b (P-TEFb) complex leads to phosphorylation of the C-terminal domain of RNA polymerase II and efficient elongation.

1.5.1. HIV-1 activation by cellular and viral transcription factors

In the following paragraphs I will describe in which way several cellular and viral transcription factors are involved during the early phase of HIV-1 gene transcription.

1.5.1.1. Sp1

Transcription factors of the Sp1 multigene family modulate HIV-1 gene transcription by direct binding to the three GC boxes adjacent to the TATAA sequence. The Sp1 factor is one of the essential cellular proteins implicated in HIV-1 gene transcription (Sune and Garcia-Blanco, 1995). A number of studies report that the Sp1 protein can serve as an anchor for indirect binding of transcription factors. For example, a cooperative interaction between Sp1 and NF-κB, bound to the adjacent sites, is required for optimal HIV-enhancer activation (Perkins et al., 1993) and also interaction between Sp1 and Tat is required for optimal Tat transactivation. A part from the three binding sites in the LTR core promoter region there are two adjacent Sp1-binding sites located in the +724/+743 LTR region downstream of the transcription start site that also play a positive regulatory role on HIV transcription (Van Lint et al., 1997). Taken together, these observations highlight the capacity of Sp1 to stimulate transcription by acting at distinct sites within the LTR, to recruit cellular factors to the LTR, and to promote Tat-mediated transactivation.

1.5.1.2. NF-κB

Proteins of the NF-κB family are involved in the control of a variety of cellular processes, such as immune and inflammatory responses, development, cellular growth, and apoptosis. A large number of stimuli activate the NF-κB pathway. Upon induction, NF-κB is liberated from its cytoplasmic inhibitor IκB and translocated into the nucleus where it binds to the two NF-κB target sites within the LTR. A variety of NF-κB homo- and heterodimers have been shown to bind to the NF-κB sites in vitro exerting differential effects on HIV gene expression. While a NF-κB-1(p50)/RelA heterodimer is the major inducible NF-κB dimer in T cells that activates strongly HIV transcription in *in vitro* transcription assays (Fujita et al., 1992; Kretzschmar et al., 1992) a homodimer of NF-κB-1(p50) may inhibit the effects of more potent activators (Franzoso et al., 1992).

1.5.1.3. NF-AT

Given the tight correlation of HIV-1 transcriptional induction to T cell activation it is not surprising that the nuclear factor of activated T cells (NF-AT) also influences HIV-1 replication.

Similar to NF-κB, NF-AT localizes to the nucleus following cellular activation. NF-AT2 binds to the NF-κB sites of the LTR and synergizes with NF-κB and Tat in transcriptional activation and HIV-1 replication (Kinoshita et al., 1998). NF-AT proteins also interact with Ets transcription factors, which results in a cooperative activation of LTR-driven transcription (Bassuk et al., 1997). Moreover, activation of NF-AT-dependent HIV gene expression appears to be stimulated by the viral Nef protein (Manninen et al., 2000).

1.5.1.4. LEF-1

LEF-1 is described as an activator of LTR-driven transcription in T lymphocytes. Mutations in the LEF-binding site in the enhancer region of the LTR (-122/-142) inhibit the transcriptional activity of the HIV-1 enhancer in Jurkat T cells (Sheridan et al., 1995). In vitro, LEF-1 binds to this LTR-binding site in a nucleosomal context (Steger and Workman, 1997). In *in vitro* transcription assays with reconstituted chromatin it has been shown that the DNA-bending protein LEF-1 in conjunction with Ets-1 and Sp1 is able to relieve nucleosomal repression of the HIV-1 LTR (Sheridan et al., 1995).

1.5.1.5. C/EBP

Two C/EBP binding sites are centered on positions -170 and -110 of the LTR and are required for full responsiveness to C/EBP (Henderson and Calame, 1997). It is interesting that transactivation by C/EBP can bypass direct interaction to its DNA binding sites through protein-protein interactions with Sp-1, NFκB and CREB proteins (Ross et al., 2001; Ruocco et al., 1996; Schwartz et al., 2000). C/EBP has also been shown to recruit histone acetyltransferases like CBP/p300 and PCAF to the LTR (Lee et al., 2002).

1.5.1.6. Viral transactivator Tat

After the early phase of transcription further provirus activation is strongly dependent on the viral transactivator Tat. Tat associates with distinct transcription factors. The transactivating function of Tat protein requires the presence of the Sp1 and NF-κB sites and a direct interaction with the Sp1 protein (Gatignol and Jeang, 2000). Tat-dependent transcription is regulated in a cell-cycle-dependent manner. While in the G1 phase, Tat transactivation is TAR-dependent and requires a

functional Sp1-binding site; in G2, a second TAR-independent phase of Tat transactivation is observed (Kashanchi et al., 2000).

Moreover, Tat has the ability to modulate the expression of various cellular factors involved in the regulation of the HIV-1 gene transcription. Tat sets up positive upregulatory loops, which greatly superactivate HIV transcription by activating NF-κB (Demarchi et al., 1996) and upregulating several cytokine genes such as TNF-α, TNF-β, IL-2, and IL-6 (Roulston et al., 1995). Tat activates NF-κB through degradation of the inhibitor I-κBα which requires the function of the cellular interferon (IFN)-inducible protein kinase PKR (Demarchi et al., 1999).

1.5.1.7. Ets-1/USF-1

By linkerscanning and deletion mutations in the distal enhancer (-130 to -166) further binding sites have been identified that are crucial for transcriptional activation and provirus-replication (Kim et al., 1993). This enhancer region bears binding sites for several transcription factors as shown by footprint analyses with T cell extracts (Demarchi et al., 1992; Sheridan et al., 1995) and the revelation of a DNAse hypersensitive site between bp -130 and -166 (Verdin et al., 1993). The transcription factors binding to this region are USF-1, Ets-1 and LEF-1, which are highly expressed in T cells. Several studies have demonstrated a positive effect of USF on HIV-1 LTR-directed transcription (Sieweke et al., 1998; Zeichner et al., 1991). Ets-1 has also been shown to transactivate HIV-1 via this enhancer (Seth et al., 1993; Sieweke et al., 1998; Sweet et al., 1998).

In a yeast one-hybrid screen, performed to find new proteins interacting with Ets-1, the E-box binding protein USF-1 has been identified. They form a complex, consisting of an USF-1 dimer and an Ets-1 monomer. The interaction surfaces of both proteins were mapped to their DNA-binding domains by analysis of deletion mutants in *in vitro* assays. Although, several related transcription factors of the Ets and the bHLH Zip family, with highly homologous DNA-binding domains, are also expressed in T cells, the interaction between Ets-1 and USF-1 was shown to be very specific. Results of transient transfection assays with a reporter under the control of the distal enhancer of the 5' HIV-1 LTR, using mutant and wild-type Ets-1 and USF-1 constructs as transcriptional activators revealed that the transcription factors act in a cooperative manner (Sieweke et al., 1998).

As mentioned above, synergistic interaction of Ets-1 with LEF-1 was reported (Sheridan et al., 1995) whereby the architectural transcription factor LEF-1 is supposed to bend DNA and bring the Ets-1/USF-1 complex in close proximity to the promoter. Also, USF-1 could stabilize this enhancer/promoter complex by forming tetramers with Inr bound USF-1 near the transcriptional start. This folding could bring together the Ets-1/USF-1 complex on the distal enhancer with the basal transcription machinery or TAT. Thus, the Ets-1/USF-1 complex could be part of a higher order structure on the HIV-1 LTR. All this indicates, that a precise positioning of the activation domains of Ets-1 and USF-1 within the three dimensional structure of the initiation complex determines their enhancer activity.

It can be suggested that this transcription factor complex plays a pivotal role in HIV-1 replication. Especially with regards to the fact that a dominant negative Ets-1 mutant (Ets-1ΔTA) completely represses USF-1 mediated transactivation (Sieweke et al., 1998) and also represses HIV-1 replication in infected target cells up to 30 fold (Posada et al., 2000).

1.5.1.8. CBP/p300

Since the provirus gets integrated into host cell chromosomes retroviruses must conserve an ability to activate transcription from a chromatin context. Recent work has revealed that the co-activators CBP/p300 (Ott et al., 1999) together with other HATs like pCAF (Kiernan et al., 1999) and hGCN5 (Col et al., 2001) help Tat to activate transcription of integrated viral DNA and derepress the HIV-1 chromatin structure in response to histone acetylation. These co-activators have to be recruited to the promoter by interacting with DNA-binding transcription factors. Several transcription factors binding to the distal enhancer and the proximal promoter region have been shown to interact directly with CBP/p300. The most prominent is the viral transactivating protein Tat. The primary function attributed to Tat is its role in HIV-1 promoter activation. Tat binds to a nascent viral leader RNA, TAR (trans-activation-responsive region) and forms a ternary complex with p300 and PCAF. This complex formation is supposed to influence proviral transcription by several means.

It seems that the “recruited” HAT activity is necessary for the activation of integrated virus but not for unintegrated supporting a role of the complex in nucleosome remodeling (Benkirane et al., 1998). Deng et al. have shown that the Tat-p300 interaction increases the HAT activity of p300 on histone H4 that is

associated with nucleosomal DNA (Deng et al., 2001) and induces a conformational change in CBP/p300 facilitating the recruitment of proteins of the basal transcription machinery like TBP, and TFIIB.

In the context of the HIV-1 LTR several of the implicated transcription factors have been shown to be acetylated by HATs. At least three acetylation sites have been described in Tat. Among them Lysine 50 acetylated by CBP/p300 and hGCN5 influencing TAR binding and Lysine 28 acetylated by pCAF facilitating interaction with Cycline T1 and subsequent recruitment of positive transcription elongation factor (pTEFb) (Bres et al., 2002). Also cellular transcription factors such as the p50 subunit of NF-κB have been shown to be acetylated for which CBP/p300 as well as Tat is equally required. Acetylated NF-κB has an increased DNA binding capacity resulting in higher HIV-1 LTR activation. A similar effect has been observed for the TATA box binding protein (TBP) (Furia et al., 2002).

There is also evidence for a function of CBP/p300 as bridging factor. A part of all the factors described so far that potentially could serve also as pillars other viral and cellular proteins present at the HIV-1 LTR have been shown to interact with CBP/p300. For example the accessory protein Vpr that needs to recruit CBP/p300 to activate the HIV-1 LTR (Kino et al., 2002). Also, the cellular proteins Ets-1 and USF-1 have been shown to transactivate via CBP/p300 in other promoter contexts (Breen and Jordan, 2000; Yang et al., 1998), respectively).

2. Objectives

As I tried to illustrate in the introduction transcriptional control is exerted by a multitude of transcription factors binding specifically to DNA, factors recruited by DNA bound proteins and coregulatory proteins. The combination of these different factors acting on a given promoter or enhancer at a given time point and in a given environment results in the regulation of transcriptional activity. Consequently, it is important to understand how these factors “communicate” with each other and to reveal the underlying mechanisms for their activity.

2.1 General objectives

The aim of my thesis was the analysis of a transcription factor complex composed of the ETS protein family member Ets-1 and the bHLH Zip protein USF-1. It has been shown before that this complex is able to influence positively HIV-1 LTR activity and that they are cooperating in DNA binding and transactivation. However, the mechanisms leading to these functions are largely unknown. The main goal was to elucidate the molecular mechanisms for Ets-1/USF-1 cooperativity which could serve as a general model for complexes composed of other members of these same transcription factor families.

A further motivation for this project was the perspective that a deeper knowledge about the molecular mechanisms underlying Ets-1/USF-1 activity could contribute to the evaluation of this complex as a drug target to inhibit HIV-1 replication. This strategy is based on the idea that interference with cellular transcription factors should be less susceptible to the development of viral resistance than inhibitors of viral proteins. Viral mutations could only indirectly undermine the efficacy of inhibitors that disrupt the interaction of cellular transcription factors.

2.2 Specific aims

2.2.1 Ets-1/USF-1 interaction

First, I wanted to analyze Ets-1/USF-1 interaction and to narrow down the interaction surface for USF-1 in Ets-1 by biochemical methods. I performed refined deletion analysis and *in vitro* and *in vivo* interaction assays to minimize the interaction surface in Ets-1. The knowledge of the precise interaction domain proved to be valid for the further analysis of the cooperativity of Ets-1 and USF-1 in DNA-binding and transactivation.

2.2.2 Ets-1/USF-1 DNA binding

DNA-binding of Ets-1 is regulated by an autoinhibitory mechanism. Although the autoinhibitory domains are well characterized, very little is known about the regulation of autoinhibition. Previous results indicate that Ets-1/USF-1 interaction changes the DNA binding capacity of the complex. Therefore, I investigated if USF-1 was able to relieve autoinhibition of Ets-1 DNA binding and/or if Ets-1 is required for USF-1 DNA binding.

2.2.3 Ets-1/USF-1 transactivation

Ets-1 and USF-1 synergize in transactivation of a reporter gene under the control of multimerized Ets-1 and USF-1 binding elements of the HIV-1 enhancer region. What are the reasons for this cooperativity? One explanation could be the cooperative DNA binding leading to a favored DNA bound state of the complex and enhanced transactivation. I wanted to test this hypothesis and to explore if other mechanisms are responsible for the observed effect.

2.2.4 Role of CBP

Another important question is how the complex bound to a distal enhancer can influence the transcriptional machinery. Since Ets-1 has been shown to act via CBP/p300 in another context and because there is some evidence for a similar role of CBP/p300 in USF-1 transactivation I studied the role of this coactivator in Ets-1/USF-1 mediated transactivation. This allowed me to bring the Ets-1/USF-1 complex into a more global context of transcriptional control of HIV-1 transactivation.

3 Results

3.1 USF-1 interacts with Helix1 and Helix 4/5 of Ets-1 in vivo *and* in vitro

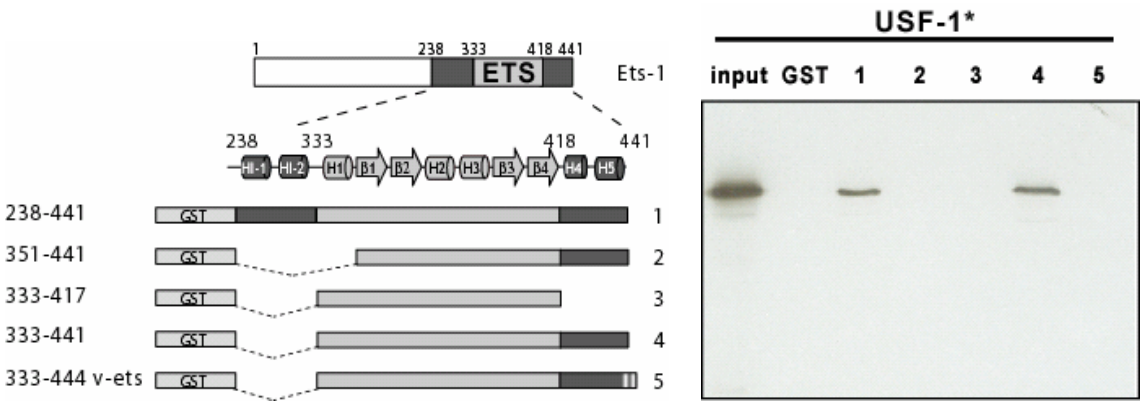
It was previously known that the main interaction domains of Ets-1 and USF-1 must be situated in their respective DNA binding domains (Sieweke 98). With the aim to identify a more restricted binding surface in Ets-1 I generated GST fused deletion constructs of the Ets-1 DNA binding domain and tested them for interaction with full length, radiolabeled *in vitro* translated USF-1 (Figure 9 A). Whereas Ets aa 280-441 and Ets aa 331-441 interact with USF-1 (lane 1+4), further deletion taking away also Helix 1 (Ets aa 351-441) or Helices 4/5 (Ets aa 333-417) that are situated N- and C-terminally of the ETS domain, respectively, result in loss of interaction (lane 2+3). Also the DBD of v-ets (333-444), a viral form of Ets-1 which differs only in the C-terminus, fails to interact with USF-1 (lane 5).

Similar results were obtained *in vivo* by a one hybrid approach in yeast. In this assay different deletion constructs of the DNA binding domain of Ets-1 bind to a LacZ reporter construct that is under control of multimerized Ets binding sites (EBS). Since all the Ets-1 constructs used lack the transactivation domain situated in the N-terminal part of the protein they are not able to transactivate the reporter alone. The potential of the different mutants to interact with USF has been tested in co-transformation experiments with the DNA binding domain of USF-1 (aa 204-310) fused to a heterologous transactivation domain derived from the VP16 protein of the herpes simplex virus (USF 204-310-VP16). Only EtsDBD (Ets 333-441) was able to recruit USF 204-310-VP16 to the promoter resulting in blue colonies due to LacZ reporter activity, whereas deletion mutants that lacked helices 4/5 (Ets 333-417) or contained the C-terminus of v-Ets (v-Ets 333-444) failed to activate the reporter construct, indicating a predominant role of the C-terminal, autoinhibitory module of Ets-1 for interaction with USF-1 (Figure 9 B)

These data indicate that Ets-1/USF-1 interaction is mediated via Helices 4/5, which correspond to the C-terminal autoinhibitory module of Ets-1, suggesting that autoinhibition and intermolecular protein interaction may be intrinsically linked.

Figure 9

A.



B.

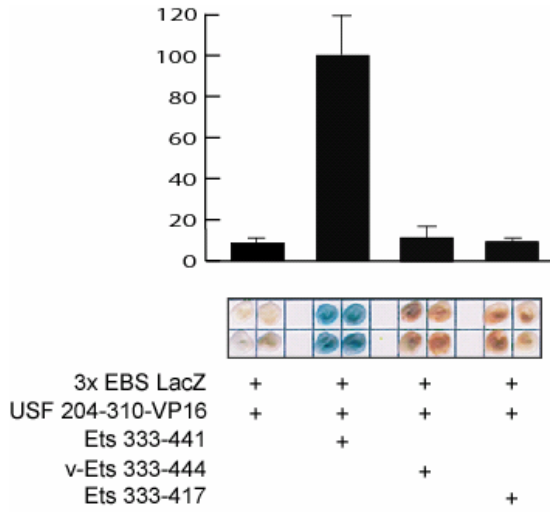


Fig.9 Mapping of a minimal interaction site in Ets-1 for USF-1. **A.** Deletion mutants of the autoinhibited DNA binding domain of Ets-1(aa238-441) fused to GST were tested for association with *in vitro* translated [35S] methionine-labeled USF. Left, maps of constructs indicating the first and the last amino acid from Ets-1 or v-ets with indications of secondary structural elements. ETS domain designates the core DNA binding domain without the inhibitory domains. **B.** *In vivo* interaction of different mutants of the Ets-1 DNA binding domain with USF-1 by a yeast one hybrid approach: The reporter plasmid with the LacZ gene under the control of 5 multimerized ETS binding sites is bound by the different Ets mutants. Interaction with USF204-310-VP16 leads to activation of the reporter gene resulting in blue colonies (lower panel). Differences in activation were measured in a quantitative ß-gal assay (upper panel).

3.2 USF-1 relieves autoinhibition of Ets-1 DNA binding through interaction with autoinhibitory Helices4/5

As discussed in the introduction, DNA binding by Ets-1 is governed by autoinhibition. Since I could show that the C-terminal autoinhibitory domain, Helix 4/5, constitutes an important interaction domain for USF-1 binding, I hypothesized that USF-1 binding to this domain in Ets-1 could be responsible for relieve of Ets-1 autoinhibition. To explore this possibility I performed electrophoretic mobility shift assays (EMSA) with a ^{32}P labeled oligo corresponding to the -138 to -170bp region of the distal enhancer of the HIV-1 LTR that contains adjacent E-box and Ets binding sites. I used purified recombinant protein corresponding to the Ets-1 DNA binding domain (Ets 333-441), Ets 238-441, encompassing the two autoinhibitory domains and to the DNA binding domain of USF-1 (USF 204-310). USF 204-310 binds in this context efficiently the probe resulting in a slower migrating complex (Figure 10 A). As expected the presence of both autoinhibitory domains present in Ets 238-441 (Hagman and Grosschedl, 1992; Lim et al., 1992) reduced DNA binding affinity of this construct leading to a dramatic difference in Ets 331-441 and 238-441 binding affinity by themselves (Figure 10 B+C, lane 1-5, lower complex). By contrast, in the presence of increasing amounts of USF 1 the autoinhibited Ets construct (Ets 238-441) readily forms ternary complexes to a comparable extent as Ets 331-441 (Figure 10 B+C, lane 2-5, upper complex). This indicates that in the absence of USF 201-310 only a small proportion of Ets 238-441 is bound to DNA and that this autoinhibition can be relieved by USF binding.

Together with the mapping of the interaction domain in Ets-1 for USF-1 to Helices 4/5, these results show that interaction of USF-1 with a restricted domain in Ets-1 interrupts the molecular interplay of the two autoinhibitory modules in Ets-1 thus enabling Ets-1 to bind DNA in a ternary complex with USF-1.

Figure 10

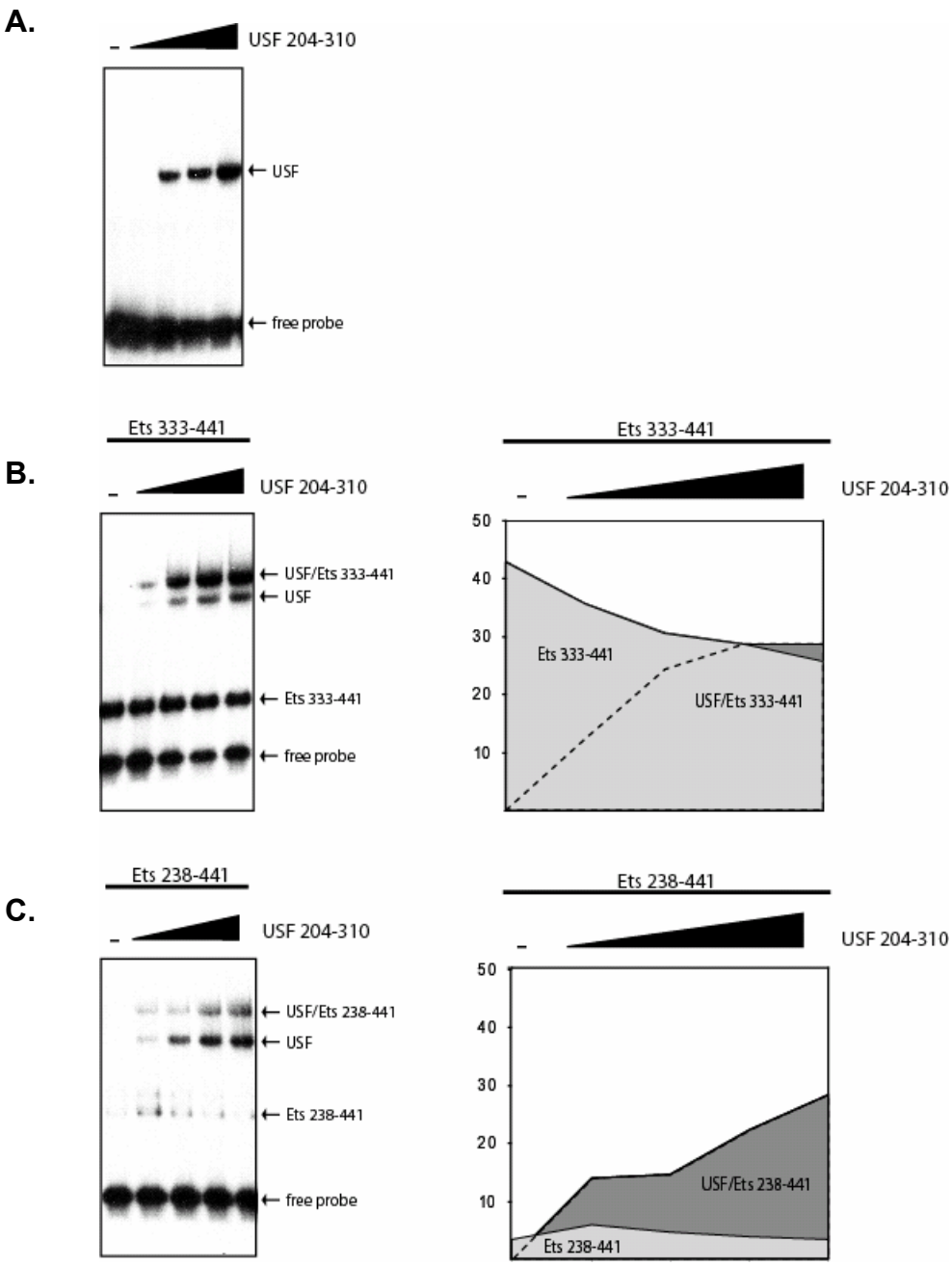


Fig.10 Cooperative DNA binding of Ets-1 and USF-1 on the distal enhancer of the HIV-1 LTR. Electrophoretic mobility shift assays (EMSA): Radiolabeled probes containing adjacent Ets-1 and USF-1 binding sites of the HIV-1 LTR were incubated with increasing amounts of recombinant purified USF-1 (USF 204-310) alone **A.** or together with either the non-autoinhibited DNA binding domain of Ets-1 (Ets 333-441) **B.** or the autoinhibited Ets 238-441 **C**. Bands corresponding to the Ets 333-441 or Ets 238-441 complex, respectively, or the ternary complex were quantified with the ImageQuant program. The graphs at the right represent the percentage of total probe bound by Ets or Ets/USF.

3.3 Cooperative transactivation by Ets-1/USF-1 complex is independent of cooperative DNA binding

We have seen before that Ets-1 and USF-1 can synergistically transactivate an HIV-1 LTR driven luciferase reporter (Sieweke et al., 1998). The interaction of USF-1 with one of the autoinhibitory domains and the resulting cooperative DNA binding described in the previous experiments could also explain the Ets-1/USF-1 cooperativity in transactivation. By consequence, higher transactivation capacity would be the result of a stabilized recruitment of the Ets-1/USF-1 complex on DNA. However, I wondered if an additional mechanism, independent of DNA binding, contributes to the observed synergistic transactivation.

To test this I decided to apply a “mammalian” hybrid system that allows to effectively uncouple DNA binding and transactivation activity of Ets-1 (see model Figure 11). Towards this end I generated fusion constructs between the DNA binding domain of the yeast protein Gal4 (Gal4DBD) and full length Ets-1 (Gal-Ets) or USF-1 (Gal-USF) to tether the transcription factors to the promoter of a Gal4 binding site driven reporter construct by a heterologous DNA binding domain. By cotransfection assays in a quail fibroblast cell line (Qt6) I tested the ability of Gal-Ets and USF-1 to synergize in reporter activation.

Both fusion constructs, Gal-Ets and Gal-USF, are able to activate expression of the luciferase reporter gene showing that they conserve their transactivation capacity when fused to the Gal4DBD. However, Gal-Ets induces much higher reporter activity (~10 fold) than Gal-USF (~1-2 fold) in comparison to the control condition. Surprisingly, addition of USF-1 to Gal-Ets increases luciferase activity by another 3 fold (Figure 11) pointing to a cooperative mechanism in transactivation independent of DNA binding by the complex. Thereby, the effect of USF-1 in cotransfections with Gal-Ets is dramatically stronger than direct recruitment of USF to DNA via the Gal moiety (compare Figure 11, conditions 2+5).

This result indicates that cooperativity in transactivation of the Ets-1/USF-1 complex is not only the result of facilitated DNA binding. It rather appears that an additional mechanism contributes to the synergism in transactivation independent of cooperative Ets-1/USF-1 DNA binding.

Figure 11

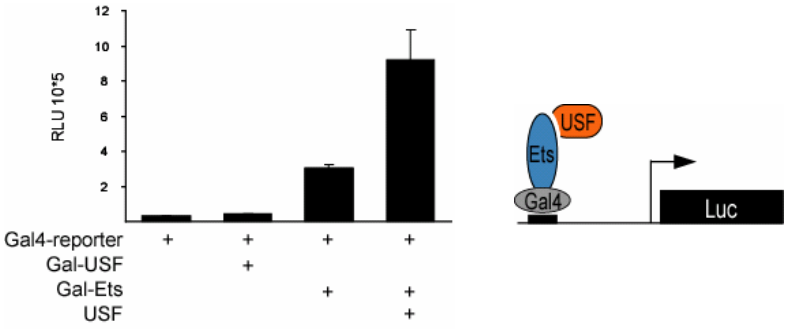


Fig.11. Ets-/USF-1 cooperativity is independent of DNA binding. Mammalian Hybrid system: Ets-1 and USF-1 are directly tethered to a luciferase gene under control of multimerized Gal4 DNA binding sites via fusion to a Gal4 DNA binding domain (Gal). The effect of interaction of free USF-1 with Gal–Ets can be measured by the reporter activity as represented by the scheme on the right. QT6 cells were co-transfected with 0.5 mg of the Gal-luciferase reporter together with the indicated expression constructs Gal-Ets (0,25 µg) and USF-1 1µg. The luciferase activities have been normalized to β-galactosidase activity from a co-transfected constitutively expressed β-gal vector. Values are expressed as relative light units and bars indicate standard errors of the mean.

3.4 Transactivation by the Ets-1/USF-1 complex depends on CBP/p300

The events described so far take place at a distant region from the promoter where the basic transcription machinery assembles. Therefore, it is important to understand by which means the Ets-1/USF-1 complex can influence the activity of gene transcription. Ets-1 has been reported to bind to the transcriptional coactivators CBP/p300 in a different context (Yang et al., 1998). Since CBP/p300 have been shown to contact transcription factors on the one hand and to modulate transcriptional activity by directly contacting the pol II machinery and/or remodeling chromatin, I wanted to investigate if reporter activation by the Ets-1/USF-1 complex also depends upon CBP/p300 thus providing the missing link between Ets-1/USF-1 and the processes at the promoter. Therefore, I applied the mammalian hybrid approach established in the previous experiments.

Again, I observed the cooperativity in transactivation independently of DNA binding by the Ets-1/USF-1 complex (Figure 12 A+B, conditions 1-3). Cotransfection of either of the two Gal4 fusion constructs, Gal-Ets and Gal-USF, respectively, with

an expression construct for CBP increased remarkably reporter activity. In the case of Gal-Ets the same level was reached as observed together with USF-1 confirming the implication of CBP in Ets-1 transactivation. Moreover, cooperative reporter activation by Gal-Ets with USF-1 could be raised by another 2 fold when cotransfected with CBP (Figure 12 A, condition 5). In the inversed setup, using Gal-USF as bait, the effect of CBP overexpression was even more striking (Figure 12 B). Cotransfection of Gal-USF with CBP alone had already a stronger effect than Ets-1 on Gal-USF and is increased by another 2-3 folds when all three elements are present.

To test whether endogenous CBP/p300 is involved in cooperative Ets-1/USF-1 transactivation I used the adenoviral protein E1A which is known to block many of the roles of CBP/p300, including their function as transcriptional coactivator (Sang et al., 2002). In cotransfection with an expression vector for E1A I tested whether activation by Gal-Ets and USF-1 is sensitive to this CBP/p300 inhibitor. Indeed activity by Gal-USF, Gal-Ets and Gal-Ets/USF could be efficiently decreased by E1A indicating that endogeneous CBP is involved in Ets-1/USF-1 transactivation (Figure 12 C).

Together, these results show that Ets-1/USF-1 transactivation depends on CBP making the link between the distal binding of the transcription factor complex and the basic transcription machinery. In addition, since overexpression of CBP leads to a further increase of Ets-1/USF-1 activity these experiments could also explain the observed synergism of the transcription factor complex due to enhanced recruitment of the transcriptional integrator CBP.

Figure 12

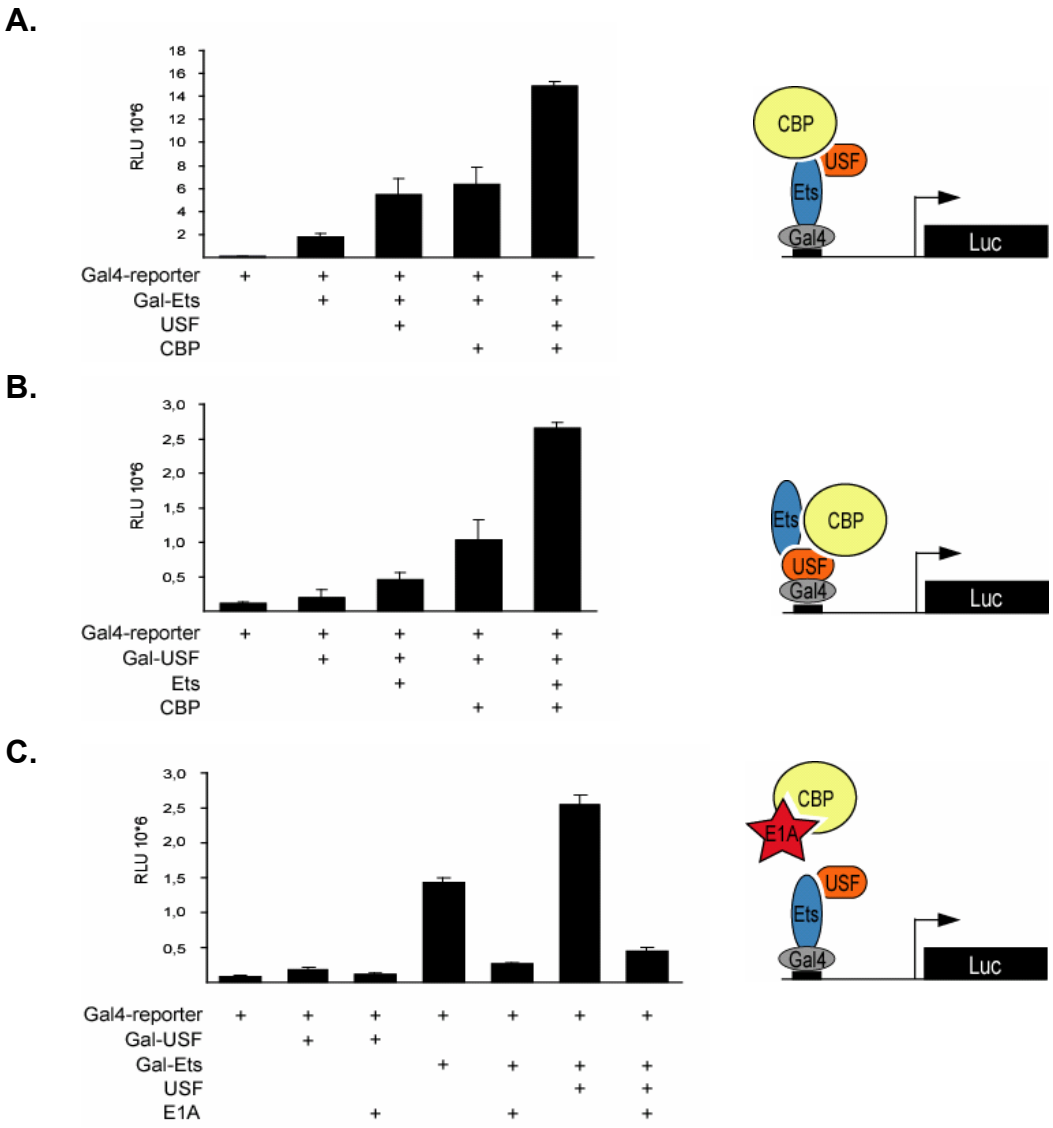


Fig.12 Influence of CBP on Ets-1/USF-1 cooperativity. Experiments were performed using the same approach described in figure 11. **A.** The Gal4 reporter (0,5µg) was cotransfected with 0,25µg of Gal-Ets and 1µg of expression constructs for USF-1 and CBP, as indicated. **B.** Same principle as in A with the inversed setup using Gal-USF as a bait. **C.** Sequestration of endogenous CBP by the adenoviral protein E1A. Cotransfection of the Gal4 luciferase reporter with 0,25 µg of expression constructs for Gal-USF, Gal-Ets ,1 µg of free Ets-1 or USF-1 and 12,5ng of E1A, as indicated.

3.5 CBP mediated synergism depends on USF-1

Since I was able to show that CBP/p300 is responsible for the synergism in transactivation by Ets-1/USF-1 I wondered how Ets-1/USF-1 complex formation can lead to enhanced CBP recruitment. Based on the knowledge that Ets-1 directly interacts with CBP I initially followed two different strategies.

First, I wanted to figure out if USF-1 could cause higher activity of Ets-1 by an induced fit mechanism which could result in increased affinity of Ets-1 for CBP.

Therefore, I tested different domains of USF-1 for their capacity to induce enhanced transactivation by Gal-Ets. I performed cotransfection studies of Gal-Ets with either the DNA binding domain of USF-1 (USF 204-310) which has no transcriptional activity by itself or its transactivation domain (USF 1-203). However, neither USF 204-310 nor USF 1-203 affect Gal-Ets activity (Figure 13 A) indicating that both parts, independently from each other, are not able to induce higher transactivation by Gal-Ets. USF 204-310 is sufficient for cooperative DNA binding (Figure 2 B+C) but not for synergistic transactivation as observed for full length USF-1. This demonstrates that regions in the more N-terminal part of USF-1 are involved in the mediation of synergism.

Secondly, to further confirm the dependence of Ets-1/USF-1 on CBP/p300 recruitment I wanted to know what would be the consequence for Ets-1/USF-1 transactivation by impairing the CBP/Ets-1 interaction. I expected that a deletion of the known interaction surface in Ets-1 for CBP/p300 (Yang et al., 1998) will abolish the observed synergism mediated by CBP.

Therefore, I generated a mutant Gal-Ets construct (Gal-Ets, Δ aa 154-211) lacking the interaction surface for CBP/p300 and performed reporter assays with Gal-Ets and Gal-EtsΔCBP in the presence or absence of full length USF-1. The deletion resulted in a loss of transactivation potential of about 50% for Gal-EtsΔCBP in comparison to Gal-Ets confirming the role of this domain for CBP mediated transactivation. However, although the overall reporter activity was a bit lower, cotransfections with USF-1 revealed an even higher synergistic effect (4,1 fold), when compared to the effect of USF-1 on Gal-Ets (2,6 fold) (Figure 13 B).

Together, these data indicate an important role of USF-1 for the mechanism underlying Ets-1/USF-1 synergism. Even though I did not observe higher activation by Gal-Ets in cotransfections with the USF domains this experiment clearly shows that regions lying in the N-terminal part of USF-1 are required for synergistic transactivation. Furthermore, the second experiment (Figure 13 B) demonstrates that synergistic activation by Ets-1/USF-1 is not exclusively dependent on Ets-1/CBP interaction. Together with the result in the previous chapter (Figure 12 B) showing increased Gal-USF transactivation potential when cotransfected CBP it is reasonable to assume that USF-1 plays a role in CBP mediated Ets-1/USF-1 synergism.

Figure 13

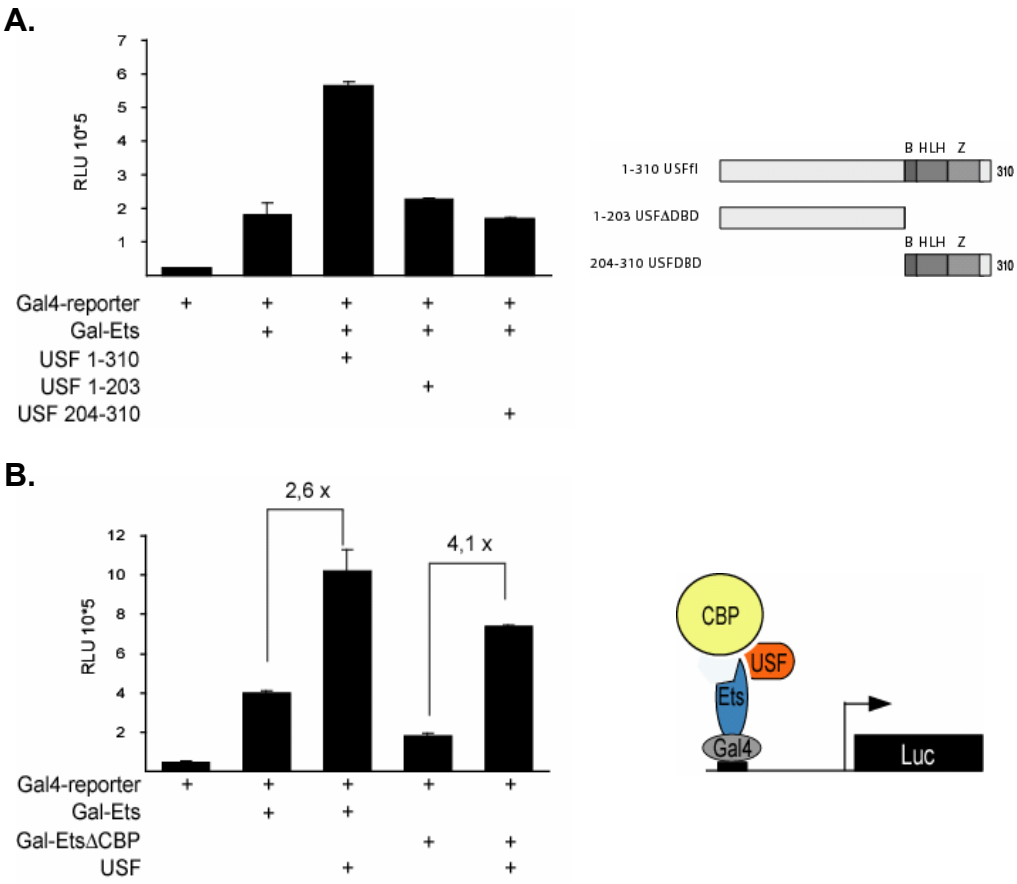


Fig.13 CBP mediated synergism depends on USF-1. **A.** Cotransfection of Gal-Ets (0,25µg) either with full length USF-1 (USF 1-310), the N-terminal domain bearing transactivation capacity (USF 1-203) or its DNA binding domain (USF 204-310) (1µg). Gal4 reporter activity was determined as described previously (see legend Fig.11). Maps of the USF-1deletion mutants are shown on the right. **B.** A Gal-Ets deletion construct was generated, lacking the CBP interaction domain (0,25µg), and cotransfected with USF-1 (1µg) and the Gal4 reporter (0,5µg).

3.6 USF-1 interacts with CBP/p300 in vitro

Since I could show that synergistic transactivation by the Ets-1/USF-1 complex is mediated by CBP and USF-1 appears to play a role in CBP recruitment I wanted to investigate if USF-1 physically interacts with CBP *in vitro.* Therefore, I performed GST-Pulldown assays.

First, I tested if radiolabeled USF-1 can interact with several GST-fusion proteins containing fragments of the ~270kD protein CBP covering almost the entire protein (Figure 14 A). As a control, also the interaction potential of Ets-1 for CBP was analyzed. As shown before, Ets-1 has been predominantly retained by GST-CBP 1-452 **(fragment 2)** bearing the CH1 domain and GST-CBP 1460-1891 **(6)** containing CH2 and to a lower extent by CBP-GST 452-721 **(3)** (Yang et al., 1998), (Figure 14

B). Under the same conditions USF-1 was also pulled down by the GST fused CBP fragments **(2)** and **(6)** (Figure 14 C). This result proves that USF-1 and CBP interact directly with each other and supports the role of CBP in Ets-1/USF-1 synergism. Interestingly, Ets-1 and USF-1 were predominantly recruited by the same fragments.

Figure 14

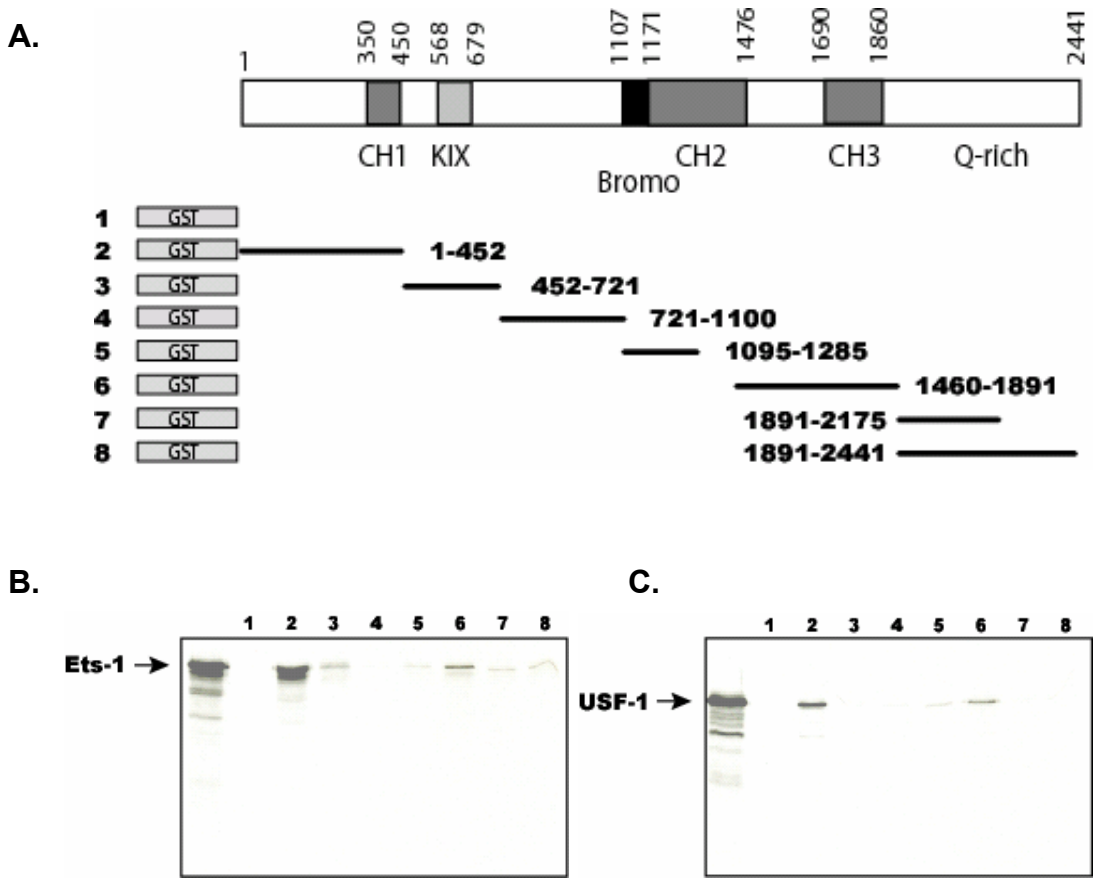


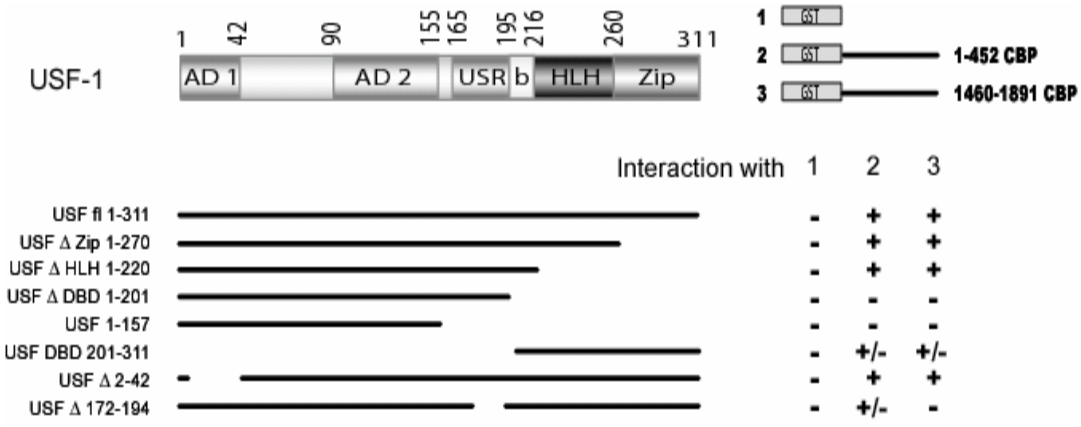
Fig.14 USF-1 interacts physially with CBP. **A.** Schematic presentation of the different GST fused CBP fragments tested for interaction with USF-1 and Ets-1. **B.** The GST fused fractions of CBP were produced in bacteria and immobilized on Sepharose beads coated with Glutathion. Equal amounts of full length Ets-1 were added in each binding reaction. The first lane corresponds to 100% of radiolabeled protein used for the binding assay. **C.** Same as in B. using in vitro translated, radiolabeled full length USF-1.

Then, I wanted to map interaction surfaces in USF-1 for CBP. Therefore, I generated truncated forms of USF-1 including internal deletions of potential transactivation domains in USF-1 which might function by recruiting a coactivator,

such as CBP/p300 (Figure 15 A). In USFΔ2-42, the activation domain 1 has been excised reported to bear transcriptional activity in *in vitro* transactivation assays (Kirschbaum et al., 1992). USFΔUSR (Δ172-194) lacks most of the USF specific region corresponding to the reported homologues regions in USF-2 which has been shown to be a powerful transactivation domain (Luo and Sawadogo, 1996).

Figure 15

A.



B.

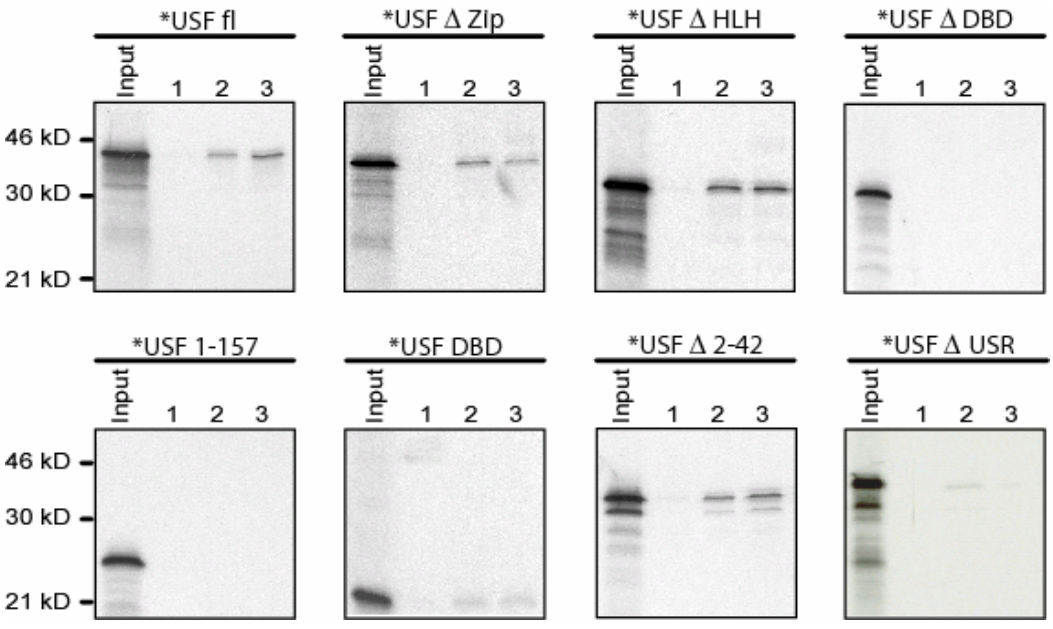


Fig.15 Mapping of an interaction surface for CBP in USF-1. **A.** Scheme of the USF-1 deletions assayed for interaction with the GST-CBP fragments shown before to bind USF-1. **B.** Presentation of the results obtained for each of the USF-1 truncations. Input corresponds to 100% of the amount of radiolabeled protein used in each condition.

These deletion mutants were tested for interaction with the GST-CBP fragments (CBP 1-452 and CBP 1460-1891) shown to interact with full length USF-1 (Figure 15 B). Truncation of the Leucine Zipper (Zip) and Helix-Loop-Helix (HLH) domains do not influence the capacity of CBP to retain USF-1. By contrast, further deletion of the basic (b) region abolishes interaction. This is consistent with the loss of interaction of USF 1-157 and USF∆DBD lacking the entire DNA binding domain. Deletion of activation domain 1 (USF ∆ 2-42) is not affected in its ability to be pulled down by both fragments. However deletion of the USR (USF ∆ 172-194) does not interact with the CBP fragment 1461-1891 and only weakly with GST-CBP 1-452. Also the DNA binding domain alone lost most of USF-1 interaction potential. It seems that the USF specific region (USR) as well as the basic region (b) are both required to mediate full USF-1/CBP interaction.

3.7 USF-1 depends on CBP/p300 for transactivation in vivo

After the identification of the interaction surface in USF-1 for CBP *in vitro* I wanted to know how this deletion would affect USF-1 activity *in vivo.* Since the basic region which I have shown to be essential for full CBP recruitment is absolutely required for USF-1 DNA binding I chose to use USF∆USR in the reporter assay rather than to delete the entire interaction surface for CBP (USR + basic region).

I performed reporter assays in Qt6 fibroblasts to compare the capacity of full length USF-1 and the USF derivates lacking the transactivation domains (USF∆2-42 and USF∆USR) to activate an HIV-1 LTR driven luciferase reporter. Whereas USF-1 and USF∆2-42 strongly induced luciferase activity, USF∆USR has almost no activity (Figure 16 A).

To confirm the dependence of USF-1 transactivation on CBP in the context of the HIV-1 LTR reporter, I tested the effect of the CBP inhibitor E1A. In cotransfection experiments USF-1 activity can be repressed by E1A but not by a mutant form (E1Amut). Consistently, the low activity that USF∆USR retains on the HIV-1 LTR reporter is not influenced by E1A confirming that the recruitment of CBP is severely impaired and results in loss of CBP mediated transactivation by USF-1 (Figure 16 B).

Figure 16

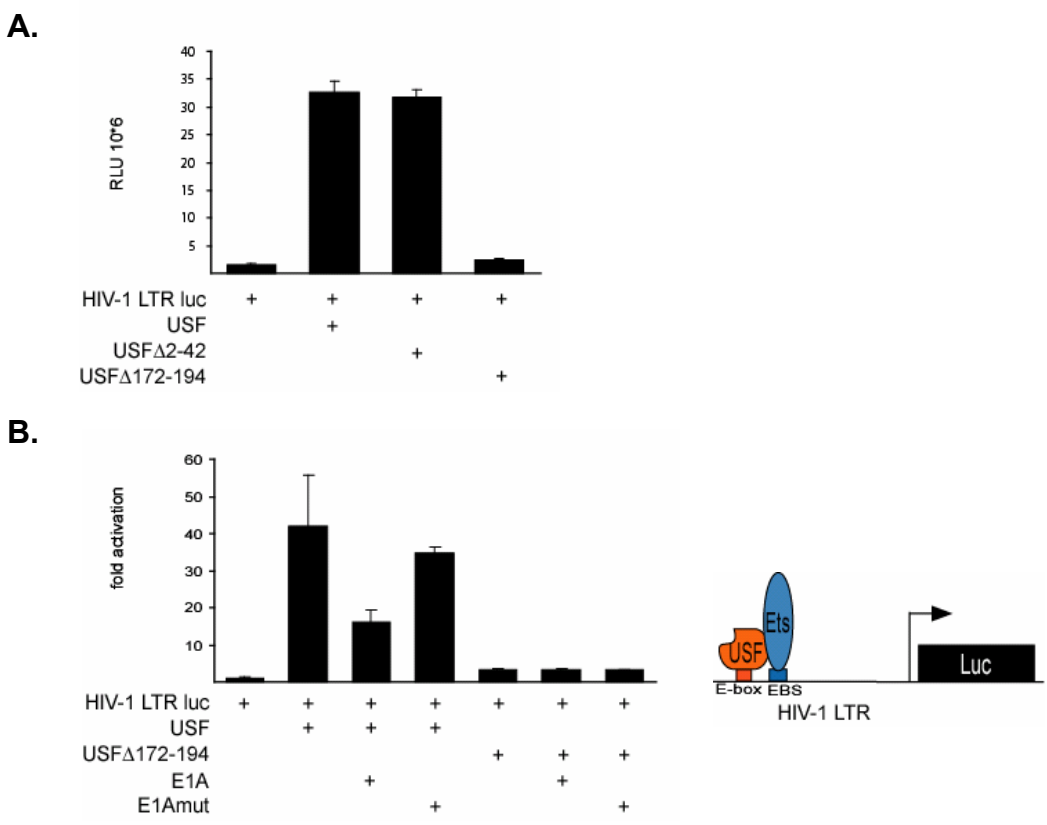


Fig.16 The USF specific region (USR, aa 172-194) is required for CBP mediated transactivation **A.** The ability of wild type USF-1 and internal deletion mutants of USF-1 (1µg), lacking either of two potential transactivation domains to transactivate a HIV-1 LTR reporter (0,5µg) was tested in Qt6 cells. **B.** Effect of E1A and E1Amut (12,5ng) on USF-1 and USFΔ172-194 mediated LTR reporter activation.

3.8 Interaction of USF-1 with CBP/p300 is required for Ets-1/USF-1 synergism

In one of the earlier experiments (Figure 13 B) I wanted to test the initial hypothesis that CBP mediated synergism depends on CBP recruitment by Ets-1. Even though the result disproved the initial hypothesis this experiment indicated a role for USF-1 in CBP mediated transactivation by Ets-1/USF-1. Since I could identify the USR in USF-1 as interaction surface for CBP which also proved to be important for USF-1 transactivation in reporter assays it became possible to evaluate the role of CBP recruitment by USF-1 for transactivation by the Ets-1/USF-1 complex.

With this aim I performed reporter assays in Qt6 cells using expression plasmids for Gal-Ets, full length USF and the USF-1 derivates lacking transactivation domains (USFΔ2-42 and USFΔUSR). As shown in figure 17, USF-1 and USFΔ2-42 synergize with Gal-Ets to activate the Gal4 driven luciferase reporter. By contrast, USFΔUSR failed to increase transcriptional activity.

This result shows that transactivation cooperativity of USF-1 and Ets 1 depends on CBP recruited by USF-1.

Figure 17

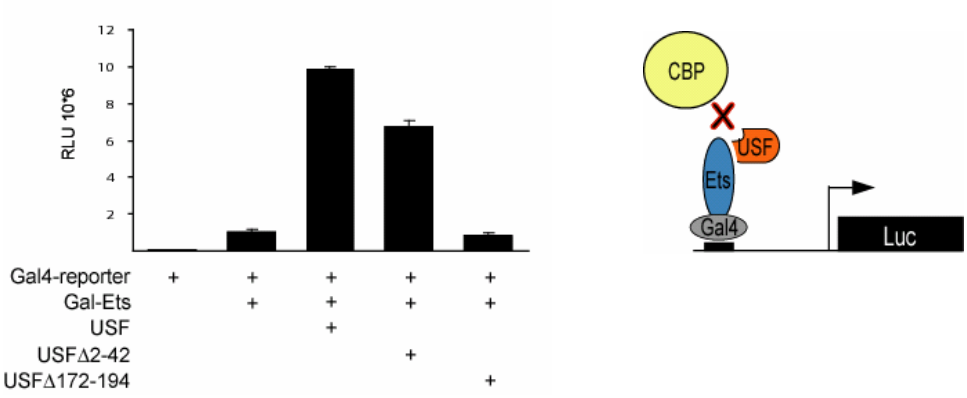


Fig.17 The USF specific region (USR, aa 172-194) is required for Ets-1/USF-1 cooperativity in transactivation. The USF-1 mutants each lacking one of USF-1 transactivation domains (USFΔ2-42 and USFΔ172-194) were analyzed for their capacity to cooperate with Gal-Ets on a Gal4 reporter in comparison to full-length USF-1.

3.9 Increased DNA binding affinity of USFΔUSR

The USF specific region is situated in close proximity to the N-terminus of the USF-1 DNA binding domain. Even though DNA binding of USFΔUSR should not be disturbed since the USF DNA binding domain (USF 204-310) retains full DNA binding capacity (see Figure 18 A), I wanted to test if the deletion of USR shows altered DNA binding properties in comparison to USF-1 (USFfl) in the context of full length proteins.

Therefore, I applied EMSA assays using *in vitro* translated, full-length USF-1 and USFΔUSR. The radioactively labeled probe used corresponds to the E-box/EBS motif used in figure 10. USF-1 recognized specifically the E-box element and the radioactive signal could be efficiently competed out by a 500 times molar excess of specific unlabeled probe but not by a nonspecific, unlabeled probe (Figure 18 A, lane 2-4). Also USFΔUSR bound specifically to the E-box element (Figure 18 A, lane 5-7). However, the deletion of the USR domain leads to higher DNA binding affinity in comparison to wild type USF-1. This strong difference in DNA binding (compare lane 2 and 5) capacity can not only be explained by the about 3-5 fold higher quantity of protein used as indicated by a Western Blot analysis of the *in vitro* translation lysates (Figure 18 B). Indeed, it appears that the affinity of full length USF-1 to the E-box is influenced by an intramolecular mechanism that involves the USF specific region (USR).

Figure 18

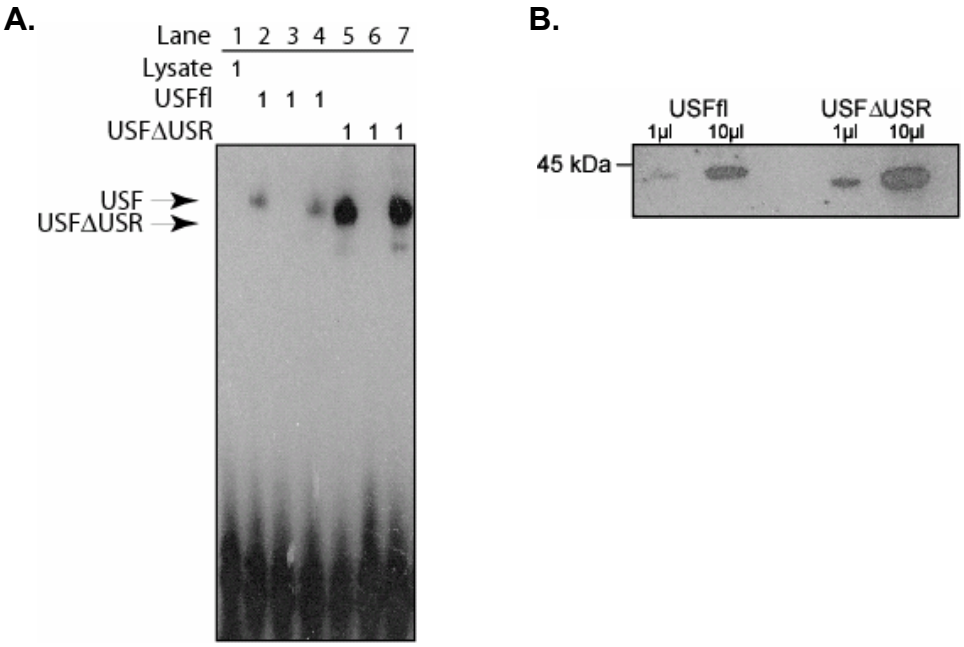


Fig.18 USFΔUSR has increased affinity for the E-box. **A.** EMSA with full length USF-1 or USFΔUSR on a radiolabeled oligonucleotide containing composite E-box/EBS binding sites. Proteins were translated in vitro. USF-1 (1µl) forms a specific DNA bound complex (lane 2) that can be efficiently competed out by a 500 molar excess of unlabeled probe (lane 3) but not by an unlabeled non-specific probe (lane 4). USFΔUSR also binds specifically the probe (lanes 5-7). **B.** Western to verify expression and quantity of the in vitro translated USF-1derivates.

3.10 Ets-1 induces enforced DNA binding by USF-1

With the indication that USF-1 DNA binding can be controlled by intramolecular interaction the question arises if Ets-1 interferes with this intramolecular mechanism and enhances the capacity of USF-1 to bind DNA. To address this question I repeated the EMSA assays to test DNA binding by in vitro translated USF-1 and USF∆USR in the presence of full length Ets-1.

As expected, Ets-1 alone is not recognizing the E-box/EBS motif of the radiolabeled probe since it is regulated by the autoinhibitory modules (Figure 19, lane 1). By contrast both USF derivates bind readily the E-box and again show a strong difference in DNA binding capacity (Figure 19, lane 2+3). However, when incubated together with Ets-1, the amount of probe bound USF-1 reaches approximately the same level as USF∆USR alone (lane 4 in comparison to lane 3). DNA binding by USF∆USR, in contrast, is not affected by Ets-1 (lane 5).

These results confirm the influence of the USR for DNA binding by USF-1 supporting an additional role of this domain as autoinhibitory module. In addition, Ets-1 seems to enhance USF-1 DNA binding and that this effect depends on the presence of the USR. Thereby, induced USF-1 binding is apparently not dependent on ternary complex formation since I did not observe a more retarded complex in the presence of Ets-1. It rather appears that Ets-1 helps USF-1 to bind to its recognition site without participating in a DNA bound complex. This is controversial to the initial observation that Ets-1 and USF-1 form a ternary complex. However, these different observations are probably due to the different setups of the experiments. Whereas I used bacterially expressed, purified protein corresponding to the respective DNA binding domains in the first setup (Figure 10) in the second full length, *in vitro* translated Ets-1 and USF-1 were used. Either in this context the complexes formed are not stable enough or the reticulocyte lysate contains another factor of the same size as Ets-1 that interacts with USF-1 and retards complex migration but is not as efficient in complex formation as Ets-1. However, a similar effect has been documented for the ability of PEA3, another ETS factor, to induce higher DNA affinity in USF-1 (Greenall et al., 2001). They observed that the ETS domain of PEA3 enhances DNA binding of USF-1 without ternary complex formation.

Figure 19

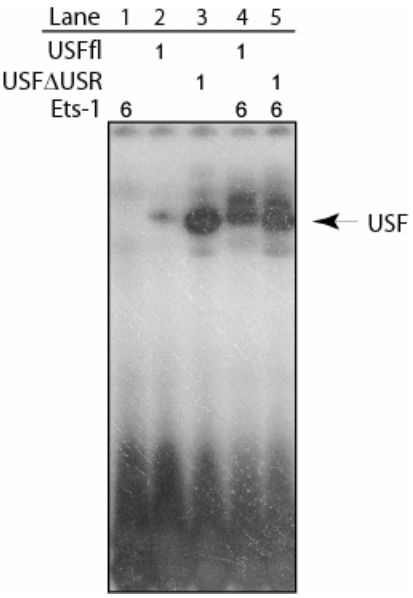


Fig.19. Ets-1 facilitates USF-1 DNA binding. **A.** EMSA with USF-1 and USFΔUSR in the absence or presence of full length Ets-1 on a radiolabeled oligonucleotide containing composite E-box/EBS binding sites. In vitro translated USF-1 and USFΔUSR (1µl) form DNA bound complexes (lanes 2+3). DNA binding capacity of USF-1 is enhanced in the presence of Ets-1 (6 µl, lane 4). The capacity of USFΔUSR to bind DNA is not changed by Ets-1 (lane5).

3.11 Ets-1 autoinhibitory domain potentates Gal-USF transactivation potential

The previous experiments underline the importance of USF-1 for the synergistic transactivation by Ets-1/USF-1 for the mediation of CBP recruitment to the complex. This contribution of USF-1 for Ets-1/USF-1 activity could be the simple result of facilitated DNA binding by the complex. However, the role of the USR in CBP recruitment and regulated DNA binding on the one hand and the ability of Ets-1 to facilitate DNA binding on the other hand propose that the interaction between Ets-1 and USF-1 could change the capacity of USF-1 to recruit CBP as well.

To analyze the effect of Ets-1 on USF-1 transactivation potential, I first inversed the setup of the mammalian hybrid experiment and generated a fusion construct between GalDBD and USF-1 (Gal-USF). In cotransfection studies with Gal-USF, the Gal responsive reporter construct and different Ets-1 derivates I observed that Ets-1 can increase transactivation by Gal-USF showing that the cooperative transactivation independent of DNA binding can be observed also in the inversed setup. Surprisingly, N-terminal truncated Ets 238-441 having no intrinsic activation potential increased Gal-USF transactivation to a similar extent. As expected, truncated Ets-1 lacking the helices 4/5 (Ets 238-417) which are important for interaction with USF-1 did not alter activation by Gal-USF (Figure 20).

This result indicates that Ets-1 induces a conformational change in USF-1 that alters the transactivation potential of Gal-USF.

Figure 20

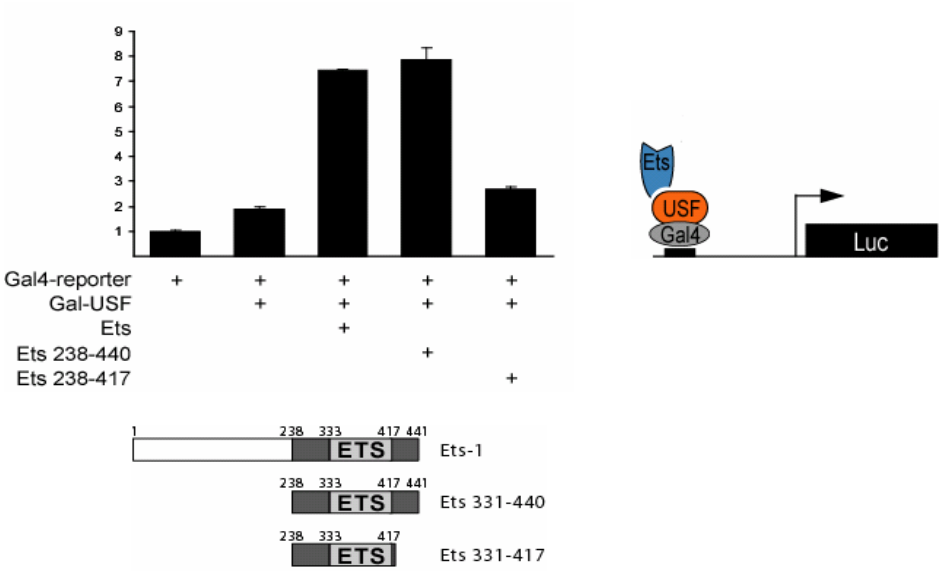


Fig.20 The C-terminal autoinhibitory domain in Ets-1 is able to induce Gal-USF transactivation. Gal-USF (0,25µg) was transfected into Qt6 cells along with Gal4 reporter plasmid and either full-length Ets-1, the autoinhibited DNA binding module of Ets-1 (Ets 238-441) or a further deletion of the C-terminal helices 4/5 (Ets 238-417). Maps of the Ets-1 deletion mutants are indicated..

4. Discussion

4.1 Summary of the results:

I could show the following:

1. USF-1 interacts with the C-terminal autoinhibitory module of Ets-1.
2. This interaction relieves autoinhibition of Ets-1 DNA binding and results in Ets-1/USF-1 complex formation at a composite binding element present in the HIV-1 LTR.
3. USF-1 DNA binding is autoinhibited by the USF specific region and Ets-1 facilitates USF-1 recruitment to the E-box.
4. Cooperative transactivation by the Ets-1/USF-1 complex is not only the result of stable recruitment of the transcription factor complex to DNA but necessitates additional events.
5. The C-terminal autoinhibitory domain of Ets-1 can induce higher transactivation capacity in USF-1.
6. CBP/p300 mediates synergistic transactivation of Ets-1/USF-1.
7. CBP/p300 mediated synergism depends on USF-1.
8. Ets-1 and USF-1 bind to the same regions in CBP.
9. The USF specific region represents the major interaction domain of USF-1 for CBP.

4.2 Discussion of the results

The results of this thesis indicate that the interplay between two transcription factors that cooperate in the control of gene expression can influence their activity by several means. In the case of the Ets-1/USF-1 complex I have shown that the interaction between the C-terminal, autoinhibitory module of Ets-1 and USF-1 provokes conformational changes that result in (1) relieve of autoinhibition of Ets-1 DNA-binding and stabilized complex recruitment to the composite binding sites that in addition is supported also by enhanced USF-1 DNA binding with the USR being involved; (2) synergism in transactivation through CBP recruitment and also by induction of transcriptional activity in USF-1 that might is due to higher accessibility of the USR domain for interaction with CBP. In the following chapters the results will be discussed more in detail.

4.2.1 Consequences of Ets-1/USF-1 complex formation

Synergistic transactivation is a frequently observed phenomenon for cooperating transcription factors. The method of choice to test the effect of cooperatively acting transcription factors on transactivation is to overexpress the factors of interest in cells. Hereby, the forced ectopic expression of a limited subset of regulatory proteins that act on a given enhancer/promoter can be sufficient to evoke synergism. This indicates that the cooperative activity of only two factors is enough to generate a greater-than-additive transcriptional activity. Given the strong effect observed by a limited part of transcription factors that are potentially implicated in the regulation of a given gene it is important to understand the molecular mechanism underlying the achievement of synergistic potential by each single transcription factor complex.

Synergism in transactivation has been shown to be the result of (1) enhanced fixation to regulatory elements in the promoter/enhancer regions and (2) subsequent recruitment of coactivators or direct contacts to the pol II machinery. These two aspects reflect on the level of transcription factor complexes the two layers of stereo-specificity underlying the enhanceosome model that tries to integrate the entity of transcription factor “signals” regulating the expression of a given gene. The Ets-1/USF-1 complex fulfills these requirements and has been shown to cooperate in DNA binding and transactivation (Sieweke et al., 1998). I now went a step further in

the analysis of the Ets-1/USF-1 activity to understand in detail the mechanistical basis for the cooperativity.

4.2.1.1 Cooperative DNA binding by Ets-1/USF-1

Many ETS-domain transcription factors are subjected to autoregulation, whereby their DNA binding activity is masked until an appropriate trigger, consisting in phosphorylation or interaction with coregulatory transcription factors is in place. Cooperative DNA binding by protein-protein interactions has been described for several members of the ETS family. For some of them exist structural evidence as for GABPα/β that are able to recognize tandem ETS binding sites after complex formation (Batchelor et al., 1998) and SAP-1/serum response factor that bind composite binding sites in the *c-fos* promoter by forming a ternary complex (Hassler and Richmond, 2001). In the example of Ets-1/Pax-5, complex formation allows the recruitment of the transcription factors to a non-consensus ETS binding site in the *mb-1* promoter. This has been shown to be achieved by conformational changes in the ETS domain induced by Pax-5 (Garvie et al., 2001). All these studies have shown that enhanced DNA binding can be reached by complex assembly resulting in additional DNA contacts and induced binding site affinity by conformational changes. By contrast, these studies did not take into account the role of the autoinhibitory domains in complex formation since they could not include these modules in the crystals.

With my biochemical approaches I could reveal that relieve of autoinhibition is triggered by direct interaction of USF-1 with one of the inhibitory modules in the case of Ets-1/USF-1. This direct interaction provides a simple and elegant mechanistic explanation for the observed cooperativity in DNA binding. A similar mechanism has been described for cooperative DNA binding by Ets-1/AML-1. Thereby, both proteins are autoregulated for DNA binding. Through interaction of the AML-1 autoregulatory domain with the N-terminal autoinhibitory module of Ets-1 they mutually relieve the block of DNA binding (Kim et al., 1999). It is remarkable that in the case of Ets-1/USF1 the other autoinhibtory module at the C-terminus is the target for the interaction partner. This proposes that apart from the functional relevance of both domains to evoke efficient inhibition of DNA binding, the modular composition of the inhibitory “intramolecular complex” also generates spatially separated interaction surfaces for distinct protein partners of Ets-1. By this strategy not only the potential

number of interaction partners is increased but hypothetically displays also the possibility to interact with two different factors at a time.

Interestingly, my finding that the capacity of USF-1 to bind to DNA is regulated as well by interaction with Ets-1 proposes a similar model to that of Ets-1/AML-1 for Ets-1/USF-1 cooperativity in DNA binding. Although interaction of Helix4/5 does not require USF-1 USR it can not be excluded that it contributes to the interaction. Greenall at al. reported that a region containing the USR in USF-1 is needed to form ternary complexes with the ETS factor PEA3 (Greenall et al., 2001). This indicate the presence of a second interaction surface in USF-1 for PEA3 that might is used also by Ets-1, in addition to the known interaction surface on the USF-1 DNA binding domain.

4.2.1.2 Synergistic transactivation by Ets-1/USF-1

The initial model describing the Ets-1/USF-1 complex proposed a scenario in which USF-1 is mainly required to recruit Ets-1 to the composite binding sites and that transactivation is exerted by Ets-1. This model was based on the fact that Ets-1 is still able to synergize with USF-1 on a reporter construct driven by an E-box element and by the use of transactivation mutants of Ets-1 and USF-1 (Sieweke et al., 1998). By separating DNA binding and transactivation function I could provide evidence that both factors contribute to the synergistic transactivation and that the role of USF-1 goes far beyond its initial role as an anchor for Ets-1 recruitment. First of all, Ets-1/USF-1 synergism can be observed in the absence of direct DNA binding by Ets-1/USF-1 and USF-1 largely enhances activity of Ets-1 tethered to DNA via the heterologous Gal4 DNA-binding domain. This effect is dependent on intact full-length USF-1 excluding the possibility that USF-1 triggers Ets-1 activity through the induction of conformational changes. By contrast, mutant Ets-1 lacking the N-terminal transactivation domain (Ets 238-441) is able to trigger higher Gal-USF-1 activity. This observation is contradictory to the previous finding that this mutant can strongly repress USF-1 activity in the context of an HIV-LTR driven reporter construct (Sieweke et al., 1998). However, with the new finding I favor a model in which Ets 238-441 is titrating out the transcriptionally more active endogeneous, full length Ets-1. This effect overlaps the potential of Ets 238-441 to induce higher USF-1 activity resulting in lower reporter activity in comparison to wild type Ets-1/USF-1 synergism. This hypothesis remains to be confirmed in cells that do not express Ets-1.

In addition I could provide a link between the Ets-1/USF-1 complex and the transcriptional “readout” by showing that the synergism depends on CBP mediated transactivation. In these experiments I could also confirm the important role of USF-1 to promote transcriptional synergism independent of DNA binding, since the loss of synergistic activity by internal deletions in Ets-1 and USF-1 depriving their CBP interaction domains was much more accentuated for USFΔUSR than for EtsΔCBP. The fact that both factors bind to the same regions in CBP indicates that they form a composite interaction surface to stabilize CBP recruitment.

4.2.2 USR domain

The USR plays an important role in many of the functions of USF. It has been characterized in USF-2 as a region that contributes to transcriptional activity and to contain an additional nuclear localization signal. This activity is context-dependent, since the USR cannot function as an activation domain when transposed to another part of the molecule (Luo and Sawadogo, 1996). This proposes that the USR has a structural role. Another report by Qyang et al. has shown that this region plays an essential role in modulating cell-specific transcriptional activity. This indicates that the function of the broadly expressed USF can be regulated (Qyang et al., 1999). USF-1 is targeted by kinases in response to stress (p38 kinase) leading to increased transcriptional activity (Galibert et al., 2001) and during the cell cycle ($p34^{cdc2}$) enhancing its DNA binding affinity (Cheung et al., 1999). The latter is supposed to phosphorylate three consensus cdc2 sites in the USR domain supporting a regulatory role for the USR. The role of USR phosphorylation for USF-1 activity confirms my observations. I could show that the internal deletion of the USR leads to increased, uninhibited binding to the E-box element. This finding also points to a structural role of the USR that establishes an autoinhibitory mechanism due to intramolecular interactions that can be relieved by phosphorylation. In addition, I show that the inhibitory effect of the USR can also be influenced by protein-protein interactions as shown here for Ets-1/USF-1. Greenall et al. also observed that the ETS factor PEA3 can enhance USF-1 binding (Greenall et al., 2001). However, this seems to be independent of the USR domain since they made the same observations for full length USF-1 and USF 197-310 lacking the USR.

Besides the regulatory role of the USR in DNA binding I could show that the highly homologues USR is also implicated in USF-1 for the mediation of transcriptional activity. Deletion of this region results not only in severely reduced transactivation potential but also in the incapacity to synergize with Ets-1. I could reveal the reason for this drastic effect by the identification of the USR as a major interaction surface for the transcriptional coactivator CBP what explains the observed phenomenon of USF∆USR in transactivation. This effect is not due to impaired nuclear localization of the truncated derivate since I did not observe a major difference between wild type USF and USF∆USR in *in situ* localization studies (data not shown) similarly to the situation in USF-2 (Luo and Sawadogo, 1996). It is notable that also the basic region shows affinity for CBP and that both, the USR and the basic domain are required for full CBP binding. It is possible that the USR interacts intramolecularly with the basic region thus inhibiting USF DNA binding and recruitment of CBP at the same time. With this model one could explain the major role of the USR for USF-1 activity.

Together, besides the finding that the USR plays a pivotal role in USF-1 functioning in analogy to USF-2, I could reveal novel functions for this region consisting in efficient CBP recruitment and regulation of USF-1 DNA binding that is influenced by protein-protein interactions with other transcription factors as Ets-1.

My results suggest that transcription factors undergo significant conformational changes between active and inactive states that are required for both DNA binding and transactivation activity. In the case of the Ets-1/USF-1 transcription factor complex the protein-protein interaction provokes dramatic conformational alterations. These allow the complex to acquire full functionality by increasing their respective DNA binding site affinity through reciprocal relieve of autoinhibition and by inducing higher transactivation capacity. Since Ets-1 itself binds DNA very poorly it is conceivable that complex formation is an event that occurs before the recruitment of the complex to DNA. Based on these findings that transcription factors acquire expanded functionality by complex formation one could regard transcription factors as exchangeable and flexible building blocks for ‘holo’ complexes whose final structure and activity is induced by participating partner molecules. The situation seems to be similar to the situation for other multi-protein complexes in the cell that

are functional only after the assembly of all subunits. However, in the case of transcription factors the individual 'subunits' can engage into partnerships with a number of different other subunits that lead to transcription factor complexes with different specificities and functions in gene regulation, as proposed in the 'cocktail party' model (Sieweke and Graf, 1998).

4.2.3 The situation at the HIV-1 LTR

The 5' long terminal repeat of HIV-1 has been described to contain binding sites for several transcription factors (Pereira et al., 2000). The influence of these factors has been extensively analyzed and a number of factors like Ets-1, USF-1, NFκB, SP1, NFAT, LEF-1 and C/EBP have been shown to be required for optimal transcriptional activity during the early phase of viral transcription (see introduction). Overall, the situation correlates well with the model of the enhanceosome (Carey, 1998). Thereby the architectural transcription factor LEF-1 bends the DNA (Giese et al., 1995; Sheridan et al., 1995) bringing distantly bound transcription factors like the Ets-1/USF-1 complex in close proximity to the promoter. But also USF-1 could contribute to stabilize loop formation. USF-1 has been shown to bind to the initiator elements of the transcriptional start site in the HIV-1 LTR (Du et al., 1993) and to be able to form tetramers with USF-1 bound to a distant E-box (Ferre-D'Amare et al., 1994).

Another property of an enhanceosome consists in the formation of a common activation surface that is complementary to target surfaces on coactivators or the pol II machinery. Several lines of evidence point to an important role of CBP/p300 for HIV-1 activation. It has been shown that chromatin remodeling at the HIV LTR depends on CBP/p300 (Benkirane et al., 1998). Among the transcription factors bound to the HIV-1 LTR NFκB (Furia et al., 2002) and C/EBP (Lee et al., 2002) have been shown to depend on CBP/p300. But also the viral transactivating protein Tat engages in a complex interplay with p300 being another anchoring point and a target for acetylation in the same time (Bres et al., 2002; Deng et al., 2001). I now could show that Ets-1/USF-1 activity is mediated by CBP as well. Given the strong inhibitory effect of USFΔUSR on the HIV-1 LTR and the absence of synergism with Ets-1 it can even be assumed that this complex makes a major contribution to the CBP recruitment in the HIV LTR "enhanceosome" context.

A model emerges in which CBP/p300 is recruited by a number of different DNA-binding proteins distributed over the distal enhancer and proximal promoter where it integrates the “input” transmitted by all these interactions as well by recruitment of other HATs and acetylation of histones and some transcription factors as well as by establishing contacts to the basal transcription machinery.

In general there still remains the question how the assembly of the transcription factors and the required coregulatory proteins occurs. Our current understanding of ‘enhanceosome’ assembly is mainly based on biochemical analysis and a model has been proposed in which different factors assemble successively on regulatory sequences to build up a functional machinery. However, in the same line of reasoning that individual transcription factors form ‘holo’ complexes to acquire their specific functions it is conceivable that even higher ordered structures are assembled at defined places in the nucleus that subsequently are recruited en bloc to a given promoter or enhancer to activate it.

4.2.4. The Ets-1/USF-1 complex as anti-HIV-1 drug target

Viral replication requires transcription of the DNA-Provirus, which produces both the viral RNA genome as well as mRNA for viral proteins. Therefore, HIV gene transcription is an attractive drug target since it depends solely on cellular transcription factors during the early phase of provirus replication. A therapy based on the inhibition of HIV transactivation by targeting cellular transcription factors would not encounter the problem of resistance development observed in therapies against viral proteins as reverse transcriptase or proteases.

Despite the complexity of factors and interactions involved in proviral transcription, previous experiments have shown that interference with individual elements of this multi-factorial complex can have significant inhibitory effects on virus transcription and replication. Thus it has been shown that the impairment of the action of single transcription factors can inhibit HIV-1 LTR activation. Some strategies target the specific DNA binding sites, for example the NFκB sites (Mischiati et al., 2002) or the TAR RNA loop by peptide nucleic acids (PNA) (Kaushik et al., 2002). Another approach consists in the use of decoy DNA containing binding sites for specific factors like NFκB or even the entire LTR to prevent binding of these transcription factors to the proviral LTR (Cho-Chung et al., 1999). HIV transactivation

could also be altered by the use of an artificial zinc finger transcription factor binding specifically to SP1 sites in the HIV-1 LTR that has been fused to the transcriptional repressor domain of the Krüppel protein (Reynolds et al., 2003). Together these studies prove the validity of the general concept of the transcriptional control machinery as a potential drug target. Even though a therapy based on this strategy would not eliminate latent provirus in an infected individual it could help to avoid the virus replication and the infection of further host cells as does also the currently administered highly active antiretroviral therapy (HAART).

However, use of the strategies described above bear a high risk of side effects. PNA would interfere also with transcription factor binding in other might essential promoters; similar decoy DNA would prevent also the physiological action of transcription factors; the artificial Zn-finger could potentially bind to any other binding site in the genome.

Since Ets-1 and USF-1 can act efficiently only when they are cooperating in DNA binding and transactivation the strategy to target a very precise domain in one of the transcription factor to inhibit the specific interaction with the other represents an attractive way to impair HIV-1 activation with the perspective to only minimally influence their function in physiological cellular processes. The validity of the Ets-1/USF-1 complex is supported by the findings of Posada et al. which could show that a dominant negative derivate of Ets-1 (Ets 238-441) prevents HIV-1 replication when overexpressed in relevant target cells (Posada et al., 2000).

Therefore it is conceivable that the Ets-1/USF-1 complex makes a major contribution to the activation of the HIV-1 provirus. My findings, that Ets-1/USF-1 recruit CBP to the HIV-1 LTR may explain the strong effect of the dominant negative effect. The identification of the C-terminal, autoinhibitory module of Ets-1 as a crucial interaction domain for USF-1 may serve as a target site for small peptides or molecules that could specifically disrupt Ets-1/USF-1 interaction and consequently the positive effect of this transcription factor complex on HIV-1 activation. The question remains to which extent the early phase of HIV-1 LTR activation depends on Ets-1/USF-1 and the capacity of the other factors binding to the LTR to activate in the absence of functional Ets-1/USF-1. However, even in the case of a certain redundancy of activating transcription factors also the other regulatory proteins engage in specific protein-protein interactions thus presenting further potential targets for anti-viral therapeutics. By blocking Ets-1/USF-1 and one ore several other

components of the HIV-1 LTR it should be possible to remarkably shut down HIV-1 activity. Alternatively, the Ets-1/USF-1 target could be used as a component of the currently administered triple therapies that would inhibit an additional step in the retroviral live cycle.

5. Perspectives

With these studies we could gain detailed insights into the processes leading to specific DNA binding and activated transactivation by the transcription factor complex between Ets-1 and USF-1. Particularly the finding that Ets-1 is able to induce higher transcriptional activity in USF-1 is pointing to a further level of mutual interference between transcription factors. My data indicate that the minimal interaction domain in Ets-1, situated in the C-terminal autoinhibitory module, can induce a conformational change in USF-1. Because of the findings about the importance of the USF specific region for transcriptional activity of USF-1 I speculate that this conformational change affects this region. Even though this event still remains hypothetical we are in a favorable situation to prove this concept.

The fact that the USR as well as the C-terminal domain interaction domain in Ets-1 are situated in close proximity to the DNA binding domains of USF-1 and Ets-1, respectively, may allow us to confirm this hypothesis by structural analysis. The proximity of the domains of interest to the respective DNA binding sites of USF-1 and Ets-1 enables us to delete large parts of the proteins facilitating the generation of ternary DNA bound Ets-1/USF-1 complexes. To do this, we are in collaboration with the group of Mathias Willmanns at the EMBL Hamburg which is trying to crystallize a ternary complex comprised of the DNA binding domain of Ets-1 including the autoinhibitory domains, USFDBD including the USR and an oligonucleotide containing the composite binding sites. The resolution of the crystal structure would be of great value to confirm my hypothesis and could complement my results.

The further analysis of the observed difference in DNA binding capacity of USF-1 and USFΔUSR will also be a topic of this collaboration. If truncated derivates of USF-1 containing the DNA binding domain with or without the USR domain show a similar effect in differential DNA binding it is planed to crystallize the DNA binding domain of USF-1 containing the USR either alone or bound to DNA. The structural analysis should reveal the respective positioning of the USR domain in the free or DNA bound state and show if this domain can engage in intramolecular interactions with the DNA binding domain. These results should provide structural evidence for the regulatory mechanism influencing USF-1 DNA binding and would provide the first

structural evidence for regulated DNA binding of a basic helix-loop-helix zipper family member by autoinhibition.

Several studies point to an important role of the Ets-1/USF-1 complex in HIV-1 LTR activation. My studies deliver a detailed view about the processes that lead to the complex' activity and it could be possible to use the ensemble of information to generate inhibitors that specifically block the Ets-1/USF-1 complex. In a first attempt, it would be interesting to confirm the important contribution of Ets-1/USF-1 for HIV-1 transactivation in a Ets-1 -/- or USF1/2 -/- cellular background. This should help to determine if the Ets-1/USF-1 complex is an attractive target for anti-HIV-1 therapeutics. As a next step one could try to identify specific inhibitors of the Ets-1/USF-1 complex. Also in this case the structural data about the ternary complex would be of great interest for the design of protein-protein interaction inhibitors.

6. Materials and Methods

In vivo interaction assay in Yeast

I used a 2µg LacZ reporter plasmid with five head to tail inserted copies of the PEA3 Ets binding site of the polyoma virus enhancer (Martin et al., 1988), 5'-TCGAGC**AGGAAGT**TTCG-3', inserted into the XhoI site of pLG670Z (Guarente and Ptashne, 1981), as described (Sieweke et al., 1996). This vector allows selection of transformed yeast by the expression of a histidine marker gene. As baits, different mutants of the Ets-1 DNA binding domain (DBD) were used, coding either for the wild type Ets (aa 333-441), for the DBD of the viral form of Ets-1 (v-Ets 333-443) or an C-terminal deletion of the Ets DBD (Ets 333-417). The different constructs were generated by inserting PCR-generated fragments coding for the respective regions in $p68^{ets\text{-}1}$ between the BamHI and EcoRI sites of the galactose inducible expression vector pSD.04a (Dalton and Treisman, 1992), conferring Tryptophan auxotrophy (Trp). The different derivates of Ets DBD were tested for interaction with USF-1 by using a further expression vector based on pSD10 (Dalton and Treisman, 1992) coding for the DNA binding domain of USF-1 (204-310) fused to the transactivation domain of the herpes simplex virus protein VP16, and bearing a selection marker gene restoring Uracil (Ura) synthesis in transformed yeast. Reporter and expression vectors were cotransformed into *Saccharomyces cerevisiae* strain W303-1A (Mat**a**, ho, his3-11,15; trp1-1; ade2-1; leu2-3,112; ura3; can1-100) by a modified lithium acetate protocol (Sieweke et al., 1996) and plated on glucose plates under selective conditions (SD -histidine/tryptophane/uracile). After 2 days of growth single colonies were restreaked on selective plates. Isolated colonies from each clone were dotted on x-gal containing selective galactose-plates containing 2% Galactose, 40µg/ml and 100mM sodium phosphate buffer (pH7.0) and tested for blue color development.

Transactivation assay in yeast

From the same clones tested for blue color development lysates were prepared to analyze quantitatively β-galactosidase activity. Single colonies of each clone were inoculated in triplicate cultures in 3ml selective synthetic galactose medium (SGal-histidine/tryptophane/uracile) and incubated for 24 hours at 30°C. Cells were harvested by centrifugation, washed and permeabilized by repeated freeze/thaw cycles on dry ice and in a waterbath at 37°C, essentially as described in

(Harshman et al., 1988). The β-galactosidase activity of the lysates was tested using ONPG (Sigma) and measured at 420nm in 96-well plates by a Miroplatereader (Wallac). The enzyme activity was normalized to cell number as measured by the optical density of the cell suspension at 600nm.

Transactivation assays in a fibroblast cell line

The QT6 cell line has been derived from quail fibroblasts (Moscovici et al., 1977) and can be easily transfected by a standard calcium phosphate transfection protocol (Graham and van der Eb, 1973). They were used in all transactivation experiments. QT6 cells from an exponentially growing culture were plated at a density of $2x10^5$ cells/well in six-wells (Falcon) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% chicken serum and 1% Penicilline/Streptamycine. 24 hours later cells were transfected with 1-5µg of DNA. The calcium phosphate DNA solutions were left for 10 min to allow precipitate formation and then added to cells in 2ml culture medium. After 15 hours medium was changed and another 33 hours later cells were dislodged from the plates by incubation in 1ml of TEN buffer (40mM Tris-HCl, pH 7.5; 10mM EDTA; 150mM NaCl). Cell pellets were resuspended in 50µl of 0.1 M potassium phosphate buffer (pH 7.3) and lysed by three freeze/thaw cycles. Aliquots of these lysates were assayed for luciferase activity (de Wet et al., 1987) and (Herbomel et al., 1984) using a luminometer (Berthold and Berthold). Relative light units from luciferase activity were normalized by the assayed β-galactosidase activity from 0.5µg co-transfected, constitutively expressed RSV-β-gal plasmid (Bonnerot et al., 1987). Each data point of the Figures was obtained by averaging duplicate samples and is representative for at least 3 independent experiments.

All Gal4-DBD-fusion constructs are based on pSG424 (Sadowski and Ptashne, 1989) and fusion proteins are expressed by a SV40 promoter. Full-length human USF-1 and chicken $p68^{Ets-1}$ were fused c-terminally to Gal-DBD (1-147) by standard cloning methods. In-frame fusions were verified by sequencing. Expression constructs of $p68^{Ets-1}$, USF-1 and their various deletion mutants are based on RC/CMV (Clontech). Internal deletions were generated with the site directed mutagenesis kit (Stratagene) and checked by sequencing. Expression plasmids for CBP, E1A and E1Amut (Janknecht, 1996; Oelgeschlager et al., 1995) have been described as well as the luciferase reporter plasmid containing the wt HIV-1 LTR

(Zeichner et al., 1991) and the GAL4 luciferase reporter 17m$_2$TATAluc (Desbarats et al., 1996).

Western Blot

The expression level of unlabeled *in vitro* translated USF-1 and USFΔUSR used in EMSA assays has been determined by Western Blot. One and ten microliter of each lysate were diluted in sample buffer (50mM Tris-HCl pH 6,8 ,2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0,02% bromophenol blue) and separated by 12,5% SDS-PAGE (Harlow and Lane, 1988). Proteins were transferred from the gel to an Immobilon P membrane (Millipore) with a BioRad semi dry blotter at 2,5 mA/cm² using 25mM Tris-base, 192 mM glycine, 20% (v/v) methanol as transfer buffer. Membranes were blocked in Tris-buffered saline (TBS) with 4% w/v dry milk for 1 hour at room temperature and stained with rabbit polyclonal antibody USF (C20) (Santa Cruz, sc-229) and a secondary goat anti-rabbit peroxidase-coupled antibody (Santa Cruz, sc-2054). Antibody incubations were for 1 hour in TBS with 4% w/v dry milk followed by three washes of 15 minutes in TBS with 0,2% Triton X-100. For detection I used the ECL chemiluminiscent peroxidase kit from Amersham.

Electromobility Shift Assays (EMSA)

Expression vectors for His$_6$-tagged EtsDBD, dnEts and USFDBD are based on pET 15b plasmids (Novagen) and were described in (Lim et al., 1992; Sieweke et al., 1998). Proteins were expressed in *E.coli* strain BL21(λDE3)pLysS and purified under non-denaturating conditions by affinity chromatography on Ni++ agarose beads (Qiagen). As a probe I used double stranded oligonucleotides corresponding to the -138 to -170bp region of the HIV-1 LTR. The oligos were labeled with [γ-^{32}P]dCTP by Klenow fill-in at a concentration of <10pM (<10000 c.p.m. per reaction). Binding reactions were performed at RT for 15 min. in a total volume of 20µl containing 20mM Tris-HCl, pH 7.5; NaCl 80mM; 1mM EDTA;0.1% Triton X100; 2mM DTT; 5% Glycerol and 5µg/µl dIdC. Samples were subjected to electrophoresis on a 5% polyacrylamide gel containing 3% glycerol in a 12.5 mM Tris, 95 mM glycine buffer. Conditions for the bandshifts testing full-length USF-1 and USFΔUSR DNA binding were basically the same. However, proteins were produced in *in vitro* translations using a coupled transcription/translation kit for PCR products (TNT T7 Quick for PCR, Promega). Even though the USF proteins are encoded by plasmids I chose to use

this special kit for PCR products. The reticulocyte lysate provided probably has been deprived of exonucleases in order to protect linear PCR products from degradation. This is an important detail which made it possible to use the *in vitro* translated USF derivates in EMSA without the risk of probe degradation by exonucleases.

In vitro interaction assays

Ets protein deletion mutants were cloned into pGEX-2T (Smith, 1993) by standard methods. Constructs and GST-Ets 333-441 have been described (Sieweke et al., 1998). The amino acid junctions of GST with the Ets sequences (in bold types) are: GSPHM**GR** for Ets 238-441, GSPHMLSGSM**GPI** for Ets 333-441, Ets 333-417 and v-Ets 333-444 and GSPHMLSGSM**SYD** for Ets 351-441. GST-CBP fusion constructs were a gift from R. Janknecht and were described in (Janknecht, 1996; Oelgeschlager et al., 1995). The bacterial expression of GST fusion proteins were performed as described (Sieweke et al., 1996). The expression plasmids for the different USF-1 derivates are based on a RC/CMV vectors (Clontech) containing also a T7 promoter that allows protein translation in *in vitro* system. Deletions in USF were generated using the site directed mutagenesis kit (Stratagene) and complementary oligos hybridizing with 25 nucleotides on either site of the sequence to be deleted. In vitro translated, radiolabeled USF proteins were produced using a rabbit reticulocyte in vitro transcription/translation system (TNT/Promega) using T7 polymerase and 10µCi [^{35}S]methionine (Redivue, Amersham)/25µl reticulocyte lysate. Binding reactions were performed using 200µl binding buffer (50mM Tris-HCl, pH 7.5; NaCl 150mM; 0.05% Triton X100; PMSF/approtinin/leupeptin protease inhibitor mix) and incubated with 10µl of GST-protein loaded resin for 1h at 4°C followed by 5 washes in 1ml binding buffer. Complexes were dissolved in sample buffer, separated by PAGE (Harlow and Lane, 1988) and analyzed by Coomassie blue staining and autoradiography.

7. Abbreviations

aa	amino acid
AD	activation domain
AIDS	aquired immune deficiency syndrome
AP-1	Activator protein 1
β-Gal	beta-Galactosidase
bp	base pair
bZip	basic leucine zipper
C/EBP	CCAAT/enhancer binding protein
Ca2+	Calcium ion
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
cdk	cycline dependent kinase
CMV	cytomegalo virus (promoter)
cpm	counts per minute
CIITA	Class II transactivator
CREB	cAMP responsive element binding protein
cyc	Cycline
Δ	deletion
DBD	DNA binding domain
dIdC	deoxy Inositol-deoxy Cytosin oligomer
DMEM	Dulbecco's modified Eagle's medium
dn	dominant negative
DNA	Deoxyribonucleic acid
DTT	Dithiotrethiol
EBS	Ets binding site
ECL	enhanced chemiluminiscence
ECM	extracellular matrix
EMSA	Electrophoretic mobility shift assay
ES	Embryonic stem (cells)
ETS	E twenty six (domain)
Ets	E twenty six

FCS	fetal calf serum
fl	full length
GST	Glutathione-S-transferase
H	helix
h	hour
HAT	Histone acetyl transferase
HI	inhibitory helix
HIF	hypoxia inducible factor
HIV-1	Human immunodeficiency virus
HMG	High mobility group protein
HOX	Homeobox protein
Ig	Immunoglobulin
IL-	Interleukin-
INF	Interferon
Inr	Initiator element
kD	kilo Dalton (kDa)
LacZ	beta-Galactosidase
LTR	Long terminal repeat
Luc	Luciferase
Maf	musculoaponeurotic fibrosarcoma
MHC	Major histocompatibility complex
Mitf	microphthalmia-associated transcription factor
ml	milliliter
MLP	(adenovirus) major late promoter
mM	milliMolar
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MW	molecular weight
µg	microgram
µl	microliter
µM	microMolar
NF	nuclear factor
ng	nano gram
Ni++	Nickel ion

nm	nanometer
PAGE	Polyacrylamide gel electrophoresis
Pax	Paired box protein
PCAF	p300/CBP activating factor
PCR	Polymerase chain reaction
pH	-log[+H]
PMSF	Phenyl-methyl-sulfonyl-flouride
pol II	RNA polymerase II
p-TEFb	positive transcription-elongation factor b
PU.1	Spi-1, Sfpi-1
RLU	relative light units
RNA	ribonucleic acid
Stat	Signal transducer of activated T-cells
SV40	Simian virus 40
TA	Transactivation domain
TAF	TBP associated factor
TAR	transactivation response element
Tat	transactivating HIV protein
TBP	TATA binding protein
TCF	ternary complex factor
TCR	T-cell receptor
TNF	tumor necrosis factor
TPO	thrombopoietin
USF	upstream stimulatory factor
USR	USF specific region
v-ets	viral ets oncogene
wHTH	winged helix turn helix protein
wt	wild type
x-gal	5-bromo-4-chlore-3-indolyl-β-D-galactoside

8. References

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