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Cooperative interaction of Ets-1 with USF-1 required for HIV-1 enhancer activity in T cells

Michael H.Sieweke[1], Hildegard Tekotte, Ursula Jarosch and Thomas Graf

EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany

[1]Corresponding author
e-mail: Sieweke@EMBL-Heidelberg.de

The distal enhancer region of the human immunodeficiency virus 1 (HIV-1) long terminal repeat (LTR) is known to be essential for HIV replication and to contain immediately adjacent E-box and Ets binding sites. Based on a yeast one-hybrid screen we have identified the E-box binding protein USF-1 as a direct interaction partner of Ets-1 and found that the complex acts on this enhancer element. The binding surfaces of USF-1 and Ets-1 map to their DNA-binding domains and although these domains are highly conserved, the interaction is very selective within the respective protein family. USF-1 and Ets-1 synergize in specific DNA binding as well as in the transactivation of reporter constructs containing the enhancer element, and mutations of the individual binding sites dramatically reduce reporter activity in T cells. In addition, a dominant negative Ets-1 mutant inhibits both USF-1-mediated transactivation and the activity of the HIV-1 LTR in T cells. The inhibition is independent of Ets DNA-binding sites but requires the Ets binding surface on USF-1, highlighting the importance of the direct protein–protein interaction. Together these results indicate that the interaction between Ets-1 and USF-1 is required for full transcriptional activity of the HIV-1 LTR in T cells.

Keywords: Ets proteins/HIV-1/HLH proteins/protein–protein interaction/transcription factors

Introduction

Human immunodeficiency virus 1 (HIV-1) predominantly replicates in macrophages and helper T cells. Although this largely reflects the presence of the CD4 receptor and specific co-receptors on these cell types, viral replication requires the transcriptional activation of the integrated provirus and thus is also affected by particular combinations of tissue-specific transcription factors. Several cellular transcription factors are known to regulate the promoter/enhancer located within the U3 region of the proviral 5′ long terminal repeat (LTR; reviewed by Jones and Peterlin, 1994). For example, lymphoid specific NF-κB-type transcription factors were found to be required for virus replication in peripheral blood lymphocytes and in activated T cells (Qian *et al.*, 1994). The promoter-proximal region of the HIV-1 LTR contains two NF-κB binding sites (Ross *et al.*, 1991; Kim *et al.*, 1993) whose functional significance was demonstrated by mutational analysis in transient transfection assays (Fujita *et al.*, 1992; Kretzschmar *et al.*, 1992; Duckett *et al.*, 1993; Qian *et al.*, 1994; Lin *et al.*, 1995) and *in vitro* transcription assays (Pazin *et al.*, 1996). A variety of inflammatory and cellular stress signals lead to the activation of NF-κB and the transcription of HIV (Duh *et al.*, 1989; Israel *et al.*, 1989; Osborn *et al.*, 1989). NF-κB activity is further enhanced by synergistic transactivation with the transcription factor Sp-1 which binds to three sites adjacent to the NF-κB sites in the promoter-proximal region of the HIV-1 LTR (Figure 5A; Perkins *et al.*, 1993; Sif and Gilmore, 1994; Pazin *et al.*, 1996).

In addition to these promoter-proximal elements, a more distal enhancer region of the HIV-1 LTR has been identified as being important for transcriptional activity and viral replication in T cells. Thus, mutations in the region –130 to –166 bp completely prevent virus replication (Kim *et al.*, 1993) and reduce the activity of LTR-reporter constructs in transient transfection assays (Zeichner *et al.*, 1991). The –130 to –166 bp region of the integrated provirus also exhibits a DNase I hypersensitive site in both T cells and macrophages (Verdin *et al.*, 1993), and is protected by T cell nuclear extracts in footprint analyses (Demarchi *et al.*, 1992; Sheridan *et al.*, 1995). As shown schematically in Figure 5A, the DNA sequence in this region contains binding sites for HLH proteins (E-box) and Ets proteins (EBS), as well as for the DNA bending transcription factor LEF-1 (lymphoid enhancer binding factor-1). Both Ets-1 (Chen, 1985; Leprince *et al.*, 1988) and LEF-1 (Travis *et al.*, 1991; Waterman *et al.*, 1991) are highly expressed in T cells, and *in vitro* transcription assays with reconstituted chromatin revealed that these two factors in conjunction with Sp-1 can relieve nucleosomal repression of the HIV-1 LTR (Sheridan *et al.*, 1995). These observations suggest that the communication of Ets-1 with other transcription factors is important for the activity of the HIV-1 LTR distal enhancer region.

Direct protein–protein interactions between transcription factors are implicated in the regulation of tissue-specific gene expression, and complexes with other transcription factors have also been reported for members of the Ets family (reviewed in Ghysdael and Boureux, 1997; Graves and Petersen, 1997). To search for interaction partners of Ets-1 we employed a yeast one-hybrid screen, which we have recently developed (Sieweke *et al.*, 1996). Here we describe the identification of the E-box binding protein USF-1 as a novel Ets-1 interaction partner. The two proteins associate via their DNA-binding domains and bind cooperatively to the adjacent E-box and Ets binding sites (EBS) in the distal enhancer of the HIV-1 LTR. They also synergize in the transactivation of a reporter containing the distal enhancer element, and a dominant negative mutant of Ets-1 can inhibit both USF-1

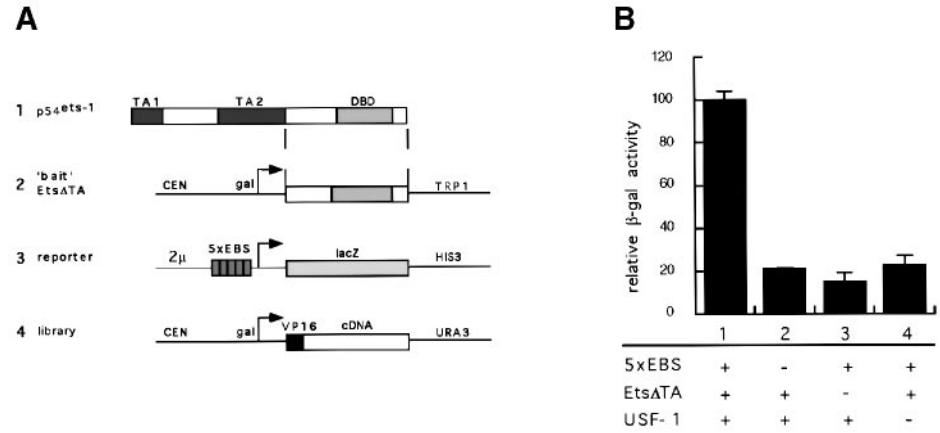

Fig. 1. Interaction of Ets-1 with USF-1 in the yeast one-hybrid assay. (**A**) Maps of constructs used in the yeast one-hybrid screen. (1) Coding region of $p54^{ets\text{-}1}$. TA1, TA2, transactivation domains; DBD, DNA binding domain. (2) Galactose-inducible 'bait' construct consisting of a truncated Ets-1 (EtsΔTA) lacking transactivation domains. (3) Reporter plasmid with the lacZ gene under the control of five multimerized Ets binding sites (EBS). (4) Galactose-inducible cDNA library expression construct tagged with a VP16 transactivation domain. TRP1, HIS3, URA3, auxotroph markers; 2μ and CEN, replication origins utilized in the plasmids. (**B**) Interaction of Ets-1 with USF-1 in the yeast one-hybrid assay. A reporter construct with or without Ets binding sites (5×EBS) was coexpressed with EtsΔTA and VP16-tagged cDNA of clone 4 (USF-1) or the corresponding empty vector in the indicated combinations. β-galactosidase activity was measured in a quantitative enzyme assay.

transactivation and HIV-1 LTR activity in T cells. These results suggest that the direct interaction of Ets-1 with USF-1 significantly contributes to the activity of the HIV-1 LTR in T cells, and explain the requirement of this enhancer region for viral replication.

Results

A yeast one-hybrid screen identifies USF-1 as an Ets-1 binding protein

We have previously described a yeast one-hybrid screen with a DNA-bound Ets-1 molecule that, unlike conventional two-hybrid systems, does not involve a fusion to a heterologous DNA-binding domain. This approach has proven successful in detecting authentic interactions with other transcription factors (Sieweke *et al.*, 1996). In brief, we used as a 'bait' an Ets-1 molecule with deleted transactivation domains which still binds specifically to DNA (EtsΔTA; Figure 1A, maps 1 and 2), and as a reporter a lacZ plasmid with five multimerized EBSs (Figure 1A, map 3). As a source of potentially interacting proteins we constructed a VP16 transactivation domain-tagged cDNA library from quail QT6 cells (Figure 1A, map 4). A library from these cells was chosen because transient transfection experiments suggested that they contain co-factors that can modulate Ets-1 activity (Lim *et al.*, 1992). Five clones were found to depend on both the library and Ets plasmids for lacZ reporter activation after a plasmid loss control experiment. Two clones (4 and 77) encoding the same gene as determined by cross-hybridizations were selected for further characterization.

To confirm that the activation of the reporter construct required a specific interaction of Ets-1 with the proteins encoded by the two library plasmids, we performed control transformations of the original yeast strain with combinations of the Ets-1 'bait', the EBS reporter and the VP16-tagged cDNA or corresponding parental control constructs. As shown in Figure 1B, lane 1, full reporter activity required the presence of Ets-1, EBS in the reporter construct and the VP16-tagged cDNA clone 4 (USF-1). Removal of either component resulted in loss of reporter activity (Figure 1B, lanes 2–4). Transformations with clone 77 yielded similar results. Sequence analysis revealed that clone 4 encodes an in-frame fusion of VP16 with 93 bp of 5′ untranslated region and the complete coding region of USF-1, a basic region helix–loop–helix–leucine zipper (bHLHZip) transcription factor. Partial sequencing of clone 77 indicated that it is identical to clone 4. The coding region of the quail cDNA from clone 4 showed 80% nucleotide and 88% amino acid identity with human USF-1 (Gregor *et al.*, 1990).

Ets-1 and USF-1 interact in solution and in vertebrate cells

To determine whether Ets-1 interacts directly with USF-1 in solution, we employed a glutathione *S*-transferase (GST) pull-down assay. For this purpose, a fusion of the C-terminus of GST with EtsΔTA was immobilized on a glutathione affinity matrix and incubated with *in vitro* translated [^{35}S]methionine-labeled human USF-1. As shown in Figure 2A, USF-1 bound to GST–EtsΔTA (lane 3), but not to GST alone (lane 4) or a GST fusion with the c-Src SH3 protein interaction domain (lane 2). Likewise, *in vitro* translated Ets-1 itself did not bind to GST–EtsΔTA (data not shown).

To test whether Ets-1 and USF-1 also interact in vertebrate cells, we transfected QT6 cells with expression constructs for human USF-1 and a His_6-tagged, truncated Ets molecule containing the region of interaction (His_6/Ets-DBD, corresponding to deletion 118 in Figure 3). This deletion was chosen instead of the full length molecule because most of the full length Ets-1 was found to be retained in the insoluble fraction under the mild lysis conditions required to preserve the interaction. After detergent lysis and sonication, an aliquot of the cell lysates was incubated with a Ni^{++} affinity matrix to precipitate the complex via the His_6-tag on Ets-1. The other aliquot of the lysates and the Ni^{++}-complexed materials were then run on an SDS–polyacrylamide gel and subjected to Western blotting using a polyclonal antibody directed

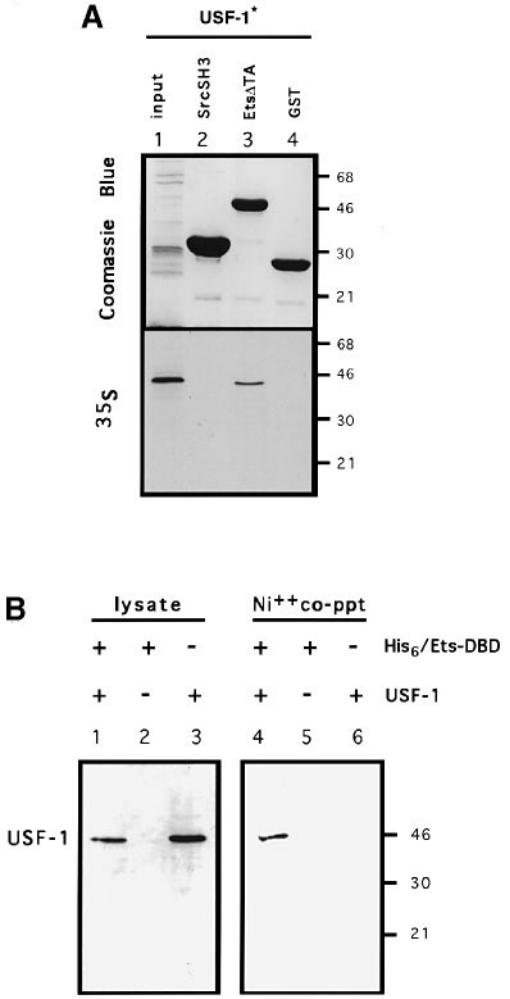

Fig. 2. Interaction of Ets-1 with USF-1 in solution and in vertebrate cells. (**A**) Interaction in solution. *In vitro* translated [^{35}S]methionine-labeled USF-1 (input, lane 1) was incubated with affinity matrix-bound GST-Src SH3 domain (lane 2), with GST-EtsΔTA (lane 3) or with GST alone (lane 4); washed, resuspended in SDS sample buffer and analyzed by SDS–PAGE. Coomassie Blue staining and autoradiography of the same gel are shown. Molecular weight markers (in kDa) are indicated on the right; the asterix indicates ^{35}S-labeled protein. (**B**) Interaction in vertebrate cells. QT6 cells were either transfected with expression constructs for a His_6-tagged DNA-binding domain of Ets-1 (His_6/Ets-DBD) together with human USF-1 (lanes 1 and 4), with His_6/Ets-DBD (lanes 2 and 5) or with human USF-1 (lanes 3 and 6) alone. Cell lysates were prepared after 48 h and either directly run on an SDS gel (lanes 1–3) or first subjected to affinity precipitation with a Ni^{++} resin (lanes 5–6). After Western transfer, blots were stained with an antibody directed against human USF-1 using the ECL technique. Molecular weight markers (in kDa) are indicated on the right.

against human USF-1. As shown in Figure 2B, USF-1 was detected in the lysates from all USF transfected cells (lanes 1 and 3) but was found in the Ni^{++}-precipitated material only when cells had been transfected with both His_6/Ets-DBD and USF-1 (lane 4), and not when they had been transfected with either of them alone (lanes 5 and 6).

The DNA-binding domain of Ets-1 binds to the bHLHZip domain of USF-1

To localize the binding surface between the interaction partners we generated deletion mutants of the two proteins. First, we constructed GST fusion proteins with various Ets deletions and tested them for binding of *in vitro* translated human USF-1 (Figure 3A). Whereas full length Ets-1 and N-terminal deletions up to the DNA-binding domain showed full binding activity (constructs 332, 106 and 118), deletions into the DNA binding domain from either the N-terminus (construct 119) or the C-terminus (constructs 116 and 117) abolished the interaction with USF-1.

We then localized the interaction surface on USF-1 by testing *in vitro* translated deletion mutants for their ability to bind to construct 106 (corresponding to EtsΔTA; Figure 3B). Deletion of the N-terminus from amino acids 1–203 up to the DNA binding domain (ΔN204) did not affect binding to Ets-1. A C-terminal deletion removing most of the leucine zipper (ΔC292) also did not affect binding activity. Two further C-terminal deletions (ΔC255 and ΔC203), however, completely abolished the interaction. Since mutant ΔC255 deletes the complete leucine zipper and most of helix 2 of the HLH domain, these results indicate that helix 2 and/or the adjacent N-terminal part of the leucine zipper helix are necessary for the interaction.

Only specific members within the ETS and bHLH families interact

Since Ets-1 and USF-1 interact via their DNA-binding domains, which are highly conserved between different members of the ETS and HLH families, we addressed the question of whether the observed interaction was restricted to Ets-1 and USF-1. Therefore, we performed pull down assays of *in vitro* translated [^{35}S]methionine-labeled USF-1 with GST fusions of different proteins containing an ETS domain. These included the DNA-binding domains of Ets-1 (Ets-DBD, corresponding to construct 118 in Figure 3), of the macrophage and B-cell specific factor PU.1 (PU.1-DBD) and of Fli-1 (Fli-1-DBD), as well as full length Elf-1. The latter two are also both expressed in T cells (Thompson *et al.*, 1992; Watson *et al.*, 1992). USF-1 bound to Ets-1 but not to PU.1 or Elf-1, and only extremely weakly to Fli-1 (Figure 4A). Under the same conditions, however, all proteins interacted equally strongly with c-Jun (data not shown).

We also tested various other *in vitro* translated HLH proteins for their ability to bind to Ets-DBD (Figure 4B). Of the bHLHZip class proteins neither c-Myc nor Mad bound to the Ets-DBD, whereas TFE-3 did, albeit more weakly than USF-1. The bHLH proteins E47, E12 and SCL/tal-1 also had no binding affinity for the Ets-1 DNA-binding domain. These results indicate that the Ets-1/USF-1 interaction is highly specific within the respective transcription factor family.

Ets-1 and USF-1 bind cooperatively to adjacent binding sites on the distal enhancer of the HIV-1 LTR

To analyze how the physical interaction of Ets-1 and USF-1 affects the DNA-binding activity of the two proteins we performed electrophoretic mobility shift assays (EMSA) with the distal enhancer of the HIV-1 LTR. For this purpose we incubated a ^{32}P-labeled oligonucleotide, corresponding to the –138 to –170 bp region of the HIV-1 LTR which encompasses adjacent E-box and Ets binding sites (EBS; Figure 5A), with purified recombinant proteins

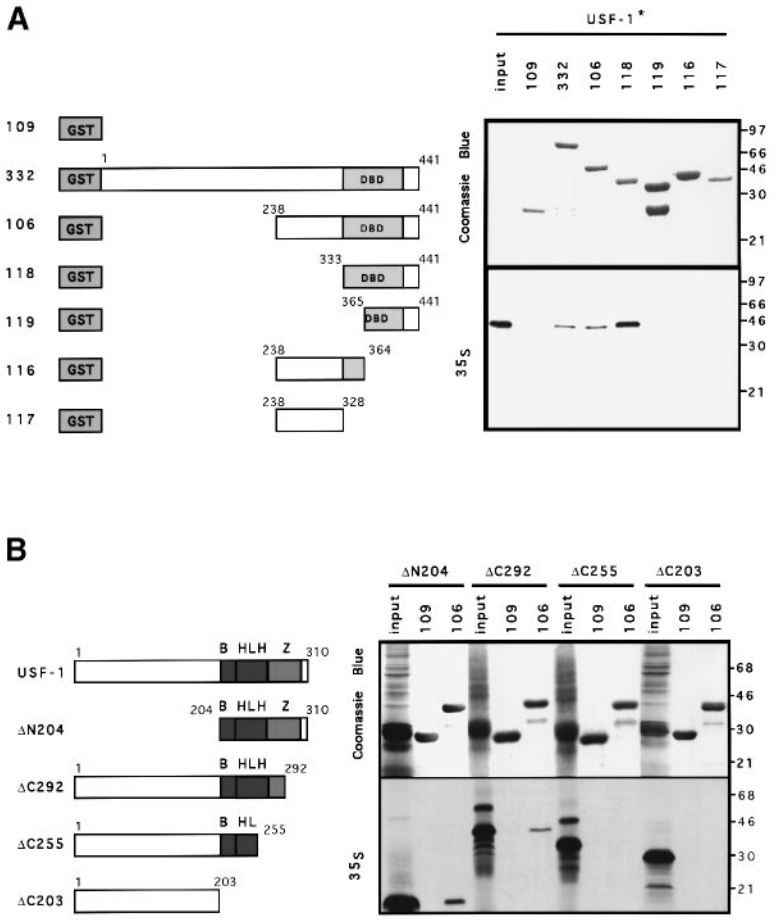

Fig. 3. Mapping of the interaction sites in Ets-1 and USF-1. (**A**) Deletion analysis of Ets-1. Full length and deletion mutants of Ets-1 fused to GST were tested for association with *in vitro* translated [^{35}S]methionine-labeled human USF-1 as described in Figure 2A. Left, maps of constructs, indicating the first and last amino acids from chicken p54^{ets-1}. Construct 106 corresponds to a GST-fusion with EtsΔTA. Right, Coomassie Blue staining (top) and autoradiography (bottom) of the same SDS–PAGE gel. (**B**) Deletion analysis of USF-1. *In vitro* translated ^{35}S-labeled deletion mutants of USF-1 were tested for association with GST–EtsΔTA (construct 106) or GST alone (construct 109) as described in Figure 2A. Left, maps of the constructs, indicating the first and last amino acids from human USF-1. B, basic region; HLH, helix–loop–helix region; Z, leucine zipper.

corresponding to the USF-1 and Ets-1 DNA-binding domains (USF-DBD and Ets-DBD, respectively). Both proteins bound specifically to the respective sites (data not shown). To assay the effects of complex formation on DNA-binding activity the oligonucleotide was incubated with increasing amounts of USF-DBD in either the absence or presence of Ets-DBD. As shown in Figure 5B, in the presence of Ets-DBD a specific ternary complex could be observed that migrated more slowly than the USF complex. Image quantification revealed a 5- to 10-fold enhancement of binding activity for the ternary complex over the USF-only complex. A similar increase in DNA-binding activity was observed with full length USF-1 (data not shown). To verify that the ternary complex contained both the Ets-DBD and USF-DBD proteins we incubated the binding reactions with specific antibodies against Ets-1 or USF-1. As shown in Figure 5C, αEts-1 IgG prevented formation of the Ets and ternary complex but not the USF complex (lane 4). Similarly, αUSF-1 IgG prevented formation of the USF and ternary complex but not the Ets complex (lane 5). Together these results indicate that Ets-1 and USF-1 can form a ternary complex on the adjacent binding sites of the distal HIV-1 LTR enhancer and that the DNA-binding activity of the complex is enhanced by protein–protein interaction.

Ets-1 and USF-1 synergistically activate the distal enhancer of the HIV-1 LTR

To address the question of whether the cooperative binding of Ets-1 and USF-1 to the –138 to –170 bp distal enhancer region of the HIV-1 LTR is also reflected in an increased transactivation potential, we inserted two copies of this element into a luciferase reporter construct in front of a thymidine kinase (tk) minimal promoter. This reporter was co-transfected together with increasing concentrations of expression constructs for USF-1 and Ets-1 into QT6 cells. As shown in Figure 6, the transactivation potential of the combined factors was significantly higher at all concentrations tested than that of the added individual activities. This effect was strongest for Ets-1, which at a concentration of 0.5 μg per dish already showed a strong synergism with each concentration of co-transfected USF-1. Similar but less dramatic results were obtained for a single copy reporter construct (data not shown). These results show that USF-1 and Ets-1 synergize on the

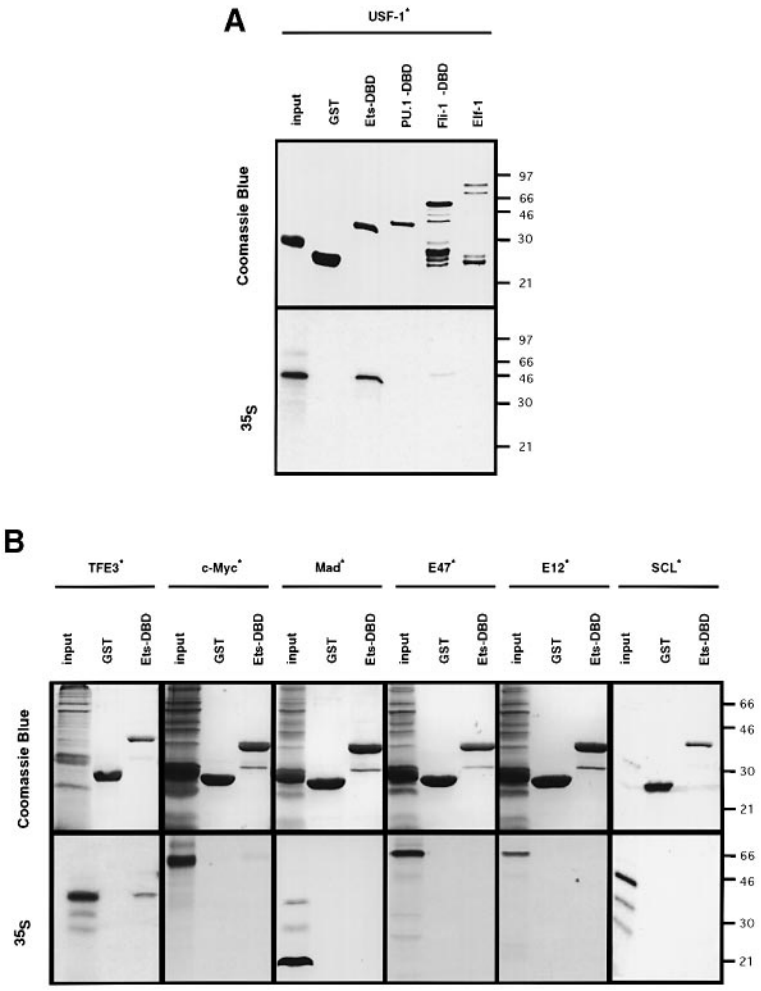

Fig. 4. Specificity of the USF-1/Ets-1 interaction. (**A**) Interaction of USF-1 with Ets family proteins. GST fusion with the DNA-binding domains of Ets-1 (Ets-DBD, construct 118), PU.1(PU.1-DBD), Fli-1 (Fli-1-DBD) and Elf-1 were tested for association with *in vitro* translated ^{35}S-labeled USF-1 as described in Figure 2A. (**B**) Interaction of Ets-1 with other bHLHZip and bHLH proteins. A GST fusion of the Ets-1 DNA-binding domain (Ets-DBD, construct 118) was tested for association with various *in vitro* translated [^{35}S]methionine-labeled bHLHZip and bHLH proteins (indicated on top) as described in Figure 2A. Coomassie Blue staining (top) and autoradiography (bottom) of the same SDS–PAGE gel are shown.

distal HIV-1 LTR enhancer, leading to a more than additive transcriptional activation of this element.

Ets- and E-box-binding activities synergize in T cells in the transactivation of the distal HIV-1 LTR enhancer element

Both Ets-1 and USF-1 are highly expressed in T cells, which are known to be primary target cells for HIV-1 infection. To determine whether, in these cells, the endogenous Ets and USF proteins also synergize on the distal enhancer of the HIV-1 LTR, we transfected Jurkat T cells with a reporter containing a single copy of the –138 to –170 bp distal enhancer element in front of a tk minimal promoter. Besides the wild-type reporter, we transfected constructs containing mutations in either the E-box or the EBS, or both. The mutations abolished USF-1 and Ets-1 binding, respectively (data not shown). As shown in Figure 7, the mutation in the EBS element caused a 60% reduction in the activity of the reporter compared with that of the wild-type element. The mutation of the E-box element had an even more severe effect (93% reduction) which was almost as strong as the double mutation (97% reduction). Furthermore, the combined activity of the reporters with single mutations is $<$50% of that of the wild-type construct, indicating that the endogenous Ets and USF activities present in T cells synergize in the transactivation of the distal enhancer element of the HIV-1 LTR. In addition, these results suggest that USF-1 can be functional in the absence of Ets-1 DNA binding, whereas Ets-1 requires DNA-bound USF-1.

A dominant negative Ets-1 protein represses USF-1 and HIV-1 LTR activity in T cells

To further investigate the significance of the interaction between USF-1 and Ets-1 for HIV-1 LTR activity, we tested the effect of transactivation defective deletion mutants of Ets-1 and USF-1 on the transactivation potential of the complex. For this purpose we used EtsΔTA (Figure 1A) which lacks transactivation domains and acts as a dominant negative mutant for Ets transactivation (data not shown). As shown in Figure 8A, EtsΔTA repressed the transactivation potential of USF-1 on the –138 to –170 bp distal enhancer element (E-box/EBS) to background levels in transient co-transfections. Surprisingly, this effect was not dependent on DNA binding of EtsΔTA, since this

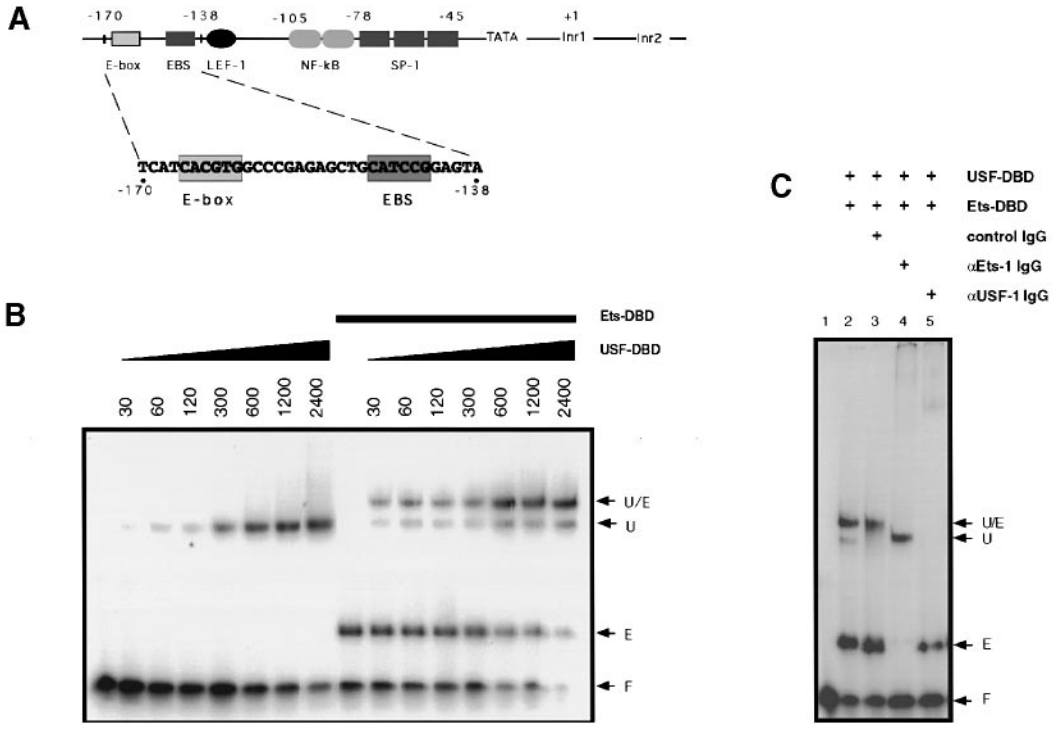

Fig. 5. Cooperative DNA binding of Ets-1 and USF-1 on the distal enhancer of the HIV-1 LTR. (**A**) Schematic drawing of the HIV-1 LTR with a detailed view of base pairs –138 to –170, containing the E-box and EBS of the distal enhancer region. Transcription factor binding sites, the TATA box and initiator elements (Inr) are indicated. (**B**) EMSAs with USF-1 DNA-binding domain on the distal enhancer in the absence or presence of Ets-1 DNA-binding domain. A radiolabeled probe of the –138 to –170 bp region of the HIV-1 LTR was incubated with increasing amounts of purified recombinant USF-1 DNA binding domain (USF-DBD; in ng) either in the absence or presence of 1.5 μg of purified recombinant Ets-1 DNA binding domain (Ets-DBD). Bands corresponding to the Ets complex (E), USF complex (U) and the ternary complex (U/E) are indicated by arrows. F, free probe. (**C**) EMSAs on the distal enhancer with purified recombinant Ets-DBD (0.6 μg) and USF-DBD (0.5 μg) in the absence (lanes 1 and 2) or presence (lanes 3–5) of 2 μg of the indicated antibodies (IgG).

construct also repressed USF-1 transactivation on a reporter with a mutated Ets binding site (E-box/EBSmut; Figure 8B). This suggests that the repressive effect of EtsΔTA can be mediated to a large extent by protein–protein interaction with USF-1. To test this hypothesis further, we performed the same experiment with the chimerical transcriptional activator GAL-USF in which the DNA-binding domain of USF-1 was replaced by the GAL4 DNA-binding domain and thus lacked the interaction surface for Ets-1. EtsΔTA had no effect on transactivation by GAL-USF, indicating that its direct interaction with the DNA-binding domain of USF-1 is required for its repressive effect (Figure 8C). In the reciprocal experiment, a deletion mutant of USF-1, competent for DNA binding but lacking the transactivation domain (USFΔTA; corresponding to deletion ΔN204 in Figure 3B), had no effect on Ets-1 transactivation (Figure 8D). This suggests that the DNA binding domain of USF-1 is sufficient to recruit a transcriptionally competent Ets-1 to the composite binding site.

To assess the importance of the USF-1/Ets-1 cooperativity in the context of the complete HIV-1 LTR, we also analyzed a full length promoter in co-transfection experiments in QT6 cells. As shown in Figure 8E, USF-1 strongly activated this construct and again EtsΔTA completely repressed this activation. Also, in this case the repression was independent of an intact Ets binding site, since a reporter with a linker scanning mutation disrupting this site (LS –130 to –147 bp) behaved like the wild-type LTR reporter.

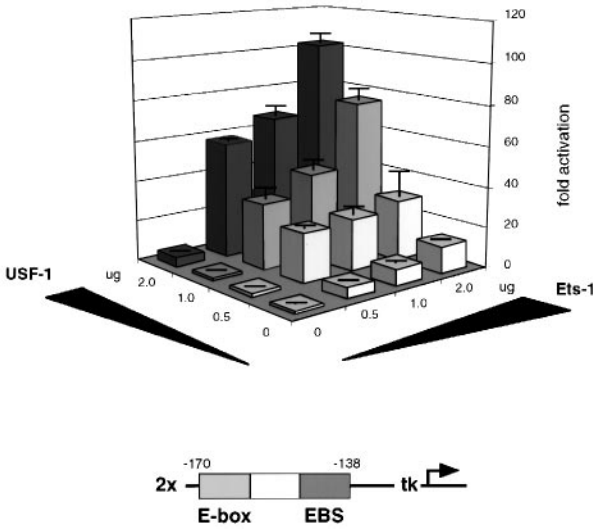

Fig. 6. Cooperative transactivation by Ets-1 and USF-1 on the distal enhancer of the HIV-1 LTR. QT6 cells were co-transfected with 0.5 μg of a luciferase reporter containing two copies of the –138 to –170 bp distal enhancer region of the HIV-1 LTR (drawn below), together with the indicated concentrations of expression constructs for Ets-1 and USF-1. Luciferase activities, normalized to β-galactosidase activity from a co-transfected lacZ vector, are expressed as fold activation and bars indicate standard errors of the mean. tk, thymidine kinase minimal promoter.

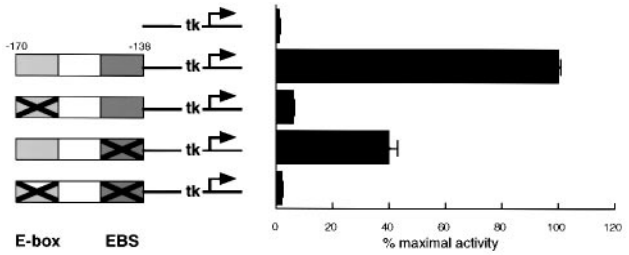

Fig. 7. Effect of E-box and EBS mutations on the activity of the HIV-1 LTR distal enhancer in T cells. Jurkat T cells were transfected with l.5 μg of a luciferase reporter construct containing a single copy of the –138 to –170 bp distal enhancer region of the HIV-1 LTR either in wild-type form, or mutated in the E-box, the EBS or both. Luciferase activities, normalized to β-galactosidase activity from a co-transfected lacZ vector, are expressed as percent of maximal activity and bars indicate standard errors of the mean.

Finally, we determined whether the dominant negative Ets protein could also repress the HIV-1 LTR in T cells. Co-transfection of EtsΔTA with the wild-type LTR reporter into Jurkat T cells resulted in a 70% repression of the HIV-1 LTR activity (Figure 9). This demonstrates that EtsΔTA not only represses exogenously overexpressed USF-1, but also the endogenous T-cell activity. Together these results suggest that the cooperation of Ets-1 and USF-1 is essential for full HIV-1 LTR activity in T cells.

Discussion

We have identified a physical and functional interaction between the winged helix–turn–helix (HTH) transcription factor Ets-1 and the bHLHZip protein USF-1. The two proteins form a specific ternary complex on the adjacent E-box and Ets binding site of the distal enhancer region of the HIV-1 LTR and synergize in both DNA binding and transcriptional activation. The interaction appears to be important for T-cell expression of the HIV-1 LTR since mutations of each individual DNA binding site drastically reduce the activity of the enhancer element in these cells, and a dominant negative Ets mutant inhibits both USF-1-mediated transactivation and HIV-1 LTR activity in T cells. Our results could thus explain the previously observed requirement of the distal enhancer region for virus replication.

Achievement of specificity through selective interactions

Various bHLH factors direct tissue-specific gene expression during the differentiation of diverse cell types including muscle, neuronal and hematopoietic cells. Since the tissue-specific bHLH factors all bind the E-box consensus sequence CANNTG with similar affinities and transactivate multimerized E-box reporters, the question arises as to what limits their action to the relevant target genes. This could be at least partially achieved by specific interactions with other transcription factors. For example, the muscle-specific transcriptional activity of the myogenic bHLH transcription factors appears to depend on their interaction with the MEF2 family of MADS-box proteins (Molkentin *et al.*, 1995). Similarly, in the hematopoietic system it has been shown that the bHLH protein SCL/tal-1 interacts with the LIM-domain protein RBTN2

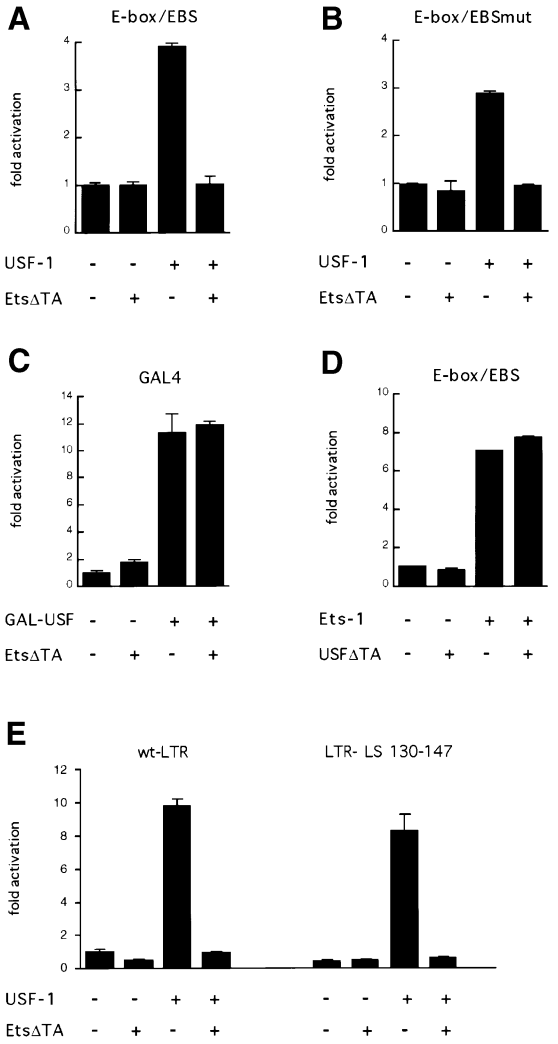

Fig. 8. Effect of transactivation domain-deleted dominant negative mutants of Ets-1 (EtsΔTA) and USF-1 (USFΔTA) on HIV-LTR distal enhancer funtion. (**A**, **B**) Effect of EtsΔTA on USF-1 transactivation of the HIV-1 LTR distal enhancer. QT6 cells were co-transfected with 0.5 μg of a luciferase reporter plasmid containing a single copy of the –138 to –170 bp distal enhancer of the HIV-1 LTR either in its wild-type form (A) or mutated in the EBS (B) together with the indicated combinations of 1 μg expression plasmids for EtsΔTA and human USF-1. (**C**) Effect of EtsΔTA on GAL–USF transactivation. QT6 cells were co-transfected with 0.5 μg of a luciferase reporter plasmid containing two GAL4 binding sites in front of a minimal TATA element and the indicated combinations of 1 μg expression plasmids for either EtsΔTA or a fusion of the GAL4 DNA-binding domain with human USF-1 lacking the bHLHZip domain (GAL–USF). (**D**) Effect of USFΔTA on Ets-1 transactivation. QT6 cells were co-transfected with 0.5 μg of a luciferase reporter plasmid containing a single copy of the –138 to –170 bp distal enhancer of the HIV-1 LTR and the indicated combinations of 1 μg expression plasmids for USFΔTA (corresponding to deletion ΔN204 in Figure 3B) and Ets-1. (**E**) Effect of EtsΔTA on USF-1 transactivation of the full length HIV-1 LTR. QT6 cells were transfected with 0.5 μg of a luciferase reporter construct containing either the full length wild-type form (left) or a linker scanning mutation of the HIV-1 LTR destroying the EBS (LS –130 to –147 bp; right) together with the indicated combinations of 1 μg expression plasmids for EtsΔTA and human USF-1. Luciferase activities, normalized to β-galactosidase activity from a co-transfected lacZ vector, are expressed as fold activation and bars indicate standard errors of the mean.

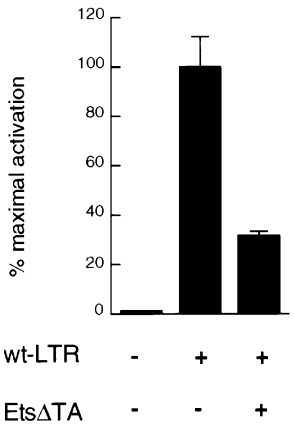

Fig. 9. Effect of EtsΔTA on HIV-1 LTR activity in T cells. Jurkat T cells were transfected with 0.2 μg of full length HIV-1 LTR reporter plasmid in the absence or presence of 2 μg EtsΔTA expression plasmid. Luciferase activities, normalized to β-galactosidase activity from a co-transfected lacZ vector, are expressed as percent of maximal activity and bars indicate standard errors of the mean.

(Osada *et al.*, 1995), which is essential for erythropoiesis (Warren *et al.*, 1994).

In the case of the bHLHZip family proteins, the need for specificity control is even more obvious since several members, including USF-1, are broadly expressed (Sirito *et al.*, 1994) and recognize the same basic E-box element, yet play a role in cell-type specific gene expression including T cells (Outram and Owen, 1994). To complicate the situation further, E-box elements are also recognized by the Myc, Max and Mad family proteins which are important for the control of cellular proliferation. Studies on the prothymosin enhancer have shown that one level of specificity control is exerted by the precise positioning of the E-box within the promoter/enhancer and the differing ability of various bHLHZip factors to act over distance (Desbarats *et al.*, 1996). Our data indicate yet another mechanism that controls the specificity of bHLHZip proteins. Despite the ability of Ets-1 to interact with several other transcription factors (see below) within the bHLH/bHLHzip family, it interacts selectively with USF-1 and to a lesser extent with TFE-3, which has also been shown to have a positive effect on Ets-1 transactivation (Sheridan *et al.*, 1995). The more restricted expression pattern of Ets-1, together with the requirement of its interaction with USF-1 for effective transactivation (see below), could thus assure that USF-1 (and possibly TFE-3) only act on a limited subset of E-boxes from which other E-box binding proteins are excluded. Interestingly, a novel zinc finger transcription factor displays an interaction profile with bHLHZip proteins that appears to be reciprocal to that of Ets-1 in that it binds to c-Myc but not to USF-1 (M.Eilers, personal communication). Selective interactions with other protein partners may thus be a general mechanism which defines the specificity of particular bHLHZip factors.

Similar to the E-box binding proteins, Ets proteins also display little difference in their DNA-binding specificity and many of them are broadly expressed (for review see Graves and Petersen, 1997). The ability of USF-1 to specifically bind Ets-1 but not other Ets family proteins expressed in T cells, like Fli-1 and Elf-1 (Thompson *et al.*, 1992; Watson *et al.*, 1992), could assure that only one, or particular Ets factors, can be recruited to the enhancer, providing a high degree of specificity.

Whereas the observed complex between Ets-1 and USF-1 is the first example of an Ets protein binding to a bHLHZip factor, transcription factors of other classes have been shown to bind to Ets-1, including the runt domain factor PEBP2α/AML-1 and the bZIP proteins c-Jun and MafB (Bassuk and Leiden, 1995; Giese *et al.*, 1995; Sieweke *et al.*, 1996). Furthermore, the paired domain protein Pax-5 can form a ternary complex with Ets-1 on the mb-1 enhancer (Fitzsimmons *et al.*, 1996). All these interactions map to the DNA-binding domain of Ets-1, found to be the target for USF-1 binding. This suggests that different classes of transcription factors have evolved an interaction surface that recognizes the same or neighboring regions on Ets-1. An interaction epitope which is specific for one or a few members within a transcription factor family, but also recognizes proteins of different DNA-binding specificity, could explain how a high degree of versatility and specificity can be achieved with a limited number of interacting factors.

Mechanism of cooperativity

Our observations indicate that the synergism of transactivation between Ets-1 and USF-1 cannot be attributed merely to cooperative DNA binding, since the Ets-DNA-binding domain which displayed cooperative DNA binding with USF-1, also repressed USF-1 transactivation similarly to the EtsΔTA mutant (data not shown). Therefore, we favor a model where the interaction of USF-1 with Ets-1 causes a synergistic presentation of their transactivation domains to the basal transcriptional machinery in addition to inducing cooperative DNA binding. Interestingly, the mutation of the DNA-binding sites (Figure 7) and the transactivation domain deletion mutants (Figure 8) have reciprocal effects on enhancer function: whereas a mutation of the E-box alone almost completely abolishes the activity of the enhancer element, USFΔTA has no effect. Conversely, the mutation of the EBS only shows a comparatively moderate effect but EtsΔTA is strongly repressive. This indicates that Ets-1 and USF-1 have complementary functions within the complex. It appears that DNA binding of USF-1 is required for correct recruitment of the complex to the enhancer element, while full length Ets-1 is necessary to establish optimal contacts with the basal transcriptional machinery. A schematic model summarizing these interpretations is shown in Figure 10.

Function within the whole promoter context

Several lines of evidence suggest that the USF-1/Ets-1 complex makes multiple contacts with promoter proximal factors and thus is part of a higher order structure on the HIV-1 LTR. Similar to the situation on the TCRα enhancer, Ets-1 synergizes with the architectural transcription factor LEF-1 on the HIV-1 LTR (Giese *et al.*, 1995; Sheridan *et al.*, 1995). This effect does not appear to depend on protein–protein interactions but rather on DNA bending induced by LEF-1, which may position Ets-1 properly to make contacts with promoter proximal factors such as Sp-1. Since Sp-1 and Ets-1 have been shown to synergize on two adjacent binding sites in the HTLV-1 LTR (Gégonne *et al.*, 1993), it is possible that they also make contacts

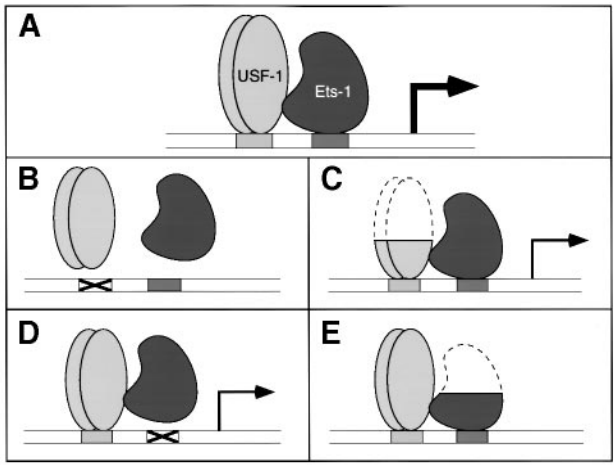

Fig. 10. Model of the USF-1/Ets-1 complex on the distal enhancer of the HIV-1 LTR. (**A**) USF-1 and Ets-1 cooperate in DNA binding and transactivation. (**B**) In the absence of an E-box, no functional complex forms. (**C**) USFΔTA enables Ets-1 recruitment and moderate transactivation. (**D**) In the absence of an EBS, USF-1 recruits Ets-1 to the enhancer, resulting in moderate transactivation. (**E**) EtsΔTA prevents USF-1 transactivation function on the distal enhancer. Light gray cassette, E-box. Dark gray cassette, EBS. Dashed lines indicate deleted transactivation domains and arrow size symbolizes transactivation strength.

on the HIV-1 LTR either by direct protein–protein interactions or through additional factors. Furthermore, we observed that the transactivation domain of Ets-1 interacts directly with TBP *in vitro*, whereas that of USF-1 does not (C.Nerlov and T.Graf, unpublished). The Ets-1/TBP interaction could thus provide another contact point that stabilizes the interaction of the Ets-1/USF-1 complex with the basal machinery.

In addition to Ets-1, USF-1 might also contribute to the interaction with promoter proximal factors. For example, it has been shown that besides the E-box in the distal enhancer analyzed in this study, USF-1 can bind to two initiator-type elements near the transcriptional start site of the HIV-1 LTR (Du *et al.*, 1993). Based on spectrophotometrical and biochemical evidence it has been proposed that USF-1 can form functional homotetramers (Ferré-D'Amare *et al.*, 1994) which could bring together an USF-1/Ets-1 complex on the distal enhancer with initiator-bound USF-1. Furthermore, interactions of USF-1 with the initiator binding factor TFII-I (Roy *et al.*, 1991) and the TBP-associated factor TAFII55 (Chiang and Roeder, 1995) have also been demonstrated, both of which may be relevant for contacts of the USF-1/Ets-1 complex with the basal machinery.

Regardless of the molecular detail of the interactions linking the USF-1/Ets-1 complex with the basal machinery, the considerations outlined above argue that the precise positioning of their transactivation domains within the three dimensional structure of the initiation complex on the HIV-1 LTR determines their enhancer activity. Our data suggest that this correct orientation of the transactivation domains depends on the interaction of Ets-1 with USF-1. Such an integration of the Ets-1/USF-1 complex in a higher order structure and its disruption by EtsΔTA would explain the observed strong inhibitory effect of EtsΔTA on the activity of the full length HIV-1 LTR.

Relevance for HIV-1 replication

The transcriptional activation of the proviral 5′ LTR constitutes an essential step in the replicative cycle of HIV-1. Deletion and linker scanning mutations affecting base pairs –130 to –166 in the distal enhancer of the HIV-1 LTR have revealed that this region is essential for viral replication in T cells and human peripheral blood lymphocytes (Kim *et al.*, 1993). This can now be explained by our observation that this region is recognized by a cooperatively acting complex of USF-1 and Ets-1. That the direct interaction of USF-1 and Ets-1 is essential for full promoter activity in T cells is indicated by the fact that a dominant negative Ets-1 mutant completely represses USF-1-mediated transactivation and strongly inhibits HIV-1 LTR activity in these cells, even when the EBS is destroyed. Consistent with this, mutations of the Ets binding site in the distal enhancer element (this study) or the full length LTR (Zeichner *et al.*, 1991) have a much weaker effect on T-cell specific activity than a mutation of the E-box. Even though USF-1 and Ets-1 bind cooperatively to adjacent sites on the DNA, it appears that in the absence of an Ets binding site Ets-1 can still be recruited to the enhancer via the interaction with USF-1. A similar situation, the recruitment of a sequence-specific DNA-binding protein to a promoter via protein interactions in the absence of a DNA-binding site, has also been reported for muscle-specific transcription factors (Molkentin and Olson, 1996).

Considering that EtsΔTA inhibited HIV-1 LTR activity more strongly than a mutation in the corresponding DNA-binding site and that even the relatively moderate effects of the latter translated into a severe repression of viral replication (Zeichner *et al.*, 1991; Kim *et al.*, 1993), we would predict that the dominant negative Ets construct will also inhibit HIV-1 replication. The disruption of protein–protein interactions between USF-1 and Ets-1 and/or other transcription factors might thus become a novel approach to inhibit the replication of HIV-1 and provide new targets for drug design.

Materials and methods

Yeast one-hybrid screen

The details of the yeast reporter strain, cDNA library construction and the interaction screen have been described previously (Sieweke *et al.*, 1996). In brief, we used a 2μ lacZ reporter plasmid with five head-to-tail ligated copies of the Ets binding site oligonucleotide 5′-TCGAGC**AGGAAGT**TTCG-3′ and galactose-inducible CEN/ARS vectors for 'bait' and library expression in the *Saccharomyces cerevisiae* strain W303-1A (Mat**a**, ho, his3-11,15; trp1-1; ade2-1; leu2-3,112; ura3; can1-100). The cDNA library was derived from a subconfluent culture of QT6 cells (Moscovici *et al.*, 1977), had a complexity of 2×10^6 and inserts ranging from 0.4–5 kb with an average of 1.6 kb. After transformation of the library into the reporter strain by a modified lithium acetate protocol (Sieweke *et al.*, 1996), yeast cells were plated on nitrocellulose filters on glucose plates with appropriate auxotroph selection. After 4 h, when small colonies appeared, the filters were transferred to galactose plates with appropriate auxotroph selection containing x-gal as a substrate for β-galactosidase. Galactose-dependent blue colonies were analyzed further by plasmid loss experiments to eliminate the clones not dependent on either the library or the bait construct. Plasmid DNA was isolated from the remaining clones by a rapid purification protocol (Hoffman and Winston, 1987) and retransformed into *Escherichia coli*. Comparison of *Hae*III restriction digest patterns and Southern blot cross hybridization (Sambrook *et al.*, 1989) of library plasmids identified three homology groups, one of which was represented by clones 4 and 77. The sequence submitted to the DDBJ/EMBL/GenBank databank was derived from

clone 4. Clone 77 had an identical insert size and *Hae*III digestion pattern, the same 5′ nucleotide sequence at the fusion with VP16 and behaved identically in interaction assays.

Quantitative β-galactosidase assays in yeast

The W303-1A strain was transformed with the indicated combination of plasmids by the modified lithium acetate method and plated under -His, -Trp and -Ura selective conditions. Single colonies were restreaked on selective plates from which triplicate cultures for each condition were inoculated in 1 ml selective synthetic galactose medium and incubated for 24 h at 30°C. Cells were harvested by centrifugation, washed, permeabilized by freezing on dry ice and assayed for β-galactosidase activity in a liquid enzyme assay using ONPG (Sigma), essentially as described (Harshman *et al.*, 1988). Enzyme activities were normalized to cell number as measured by the optical density of the cell suspension at 600 nm.

Transactivation assays

Exponentially growing quail-derived QT6 fibroblasts were plated at 2.5×10^5 cells per 35 mm plate in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS) and 1% chicken serum, and transfected 24 h later by the calcium phosphate procedure (Graham and van der Eb, 1973), allowing 20 min for precipitation and then adding 500 µl of the precipitation mix to 2 ml culture medium. Medium was changed after 4 and 18 h. After 48–72 h the cells were dislodged from the plates by incubation in 1 ml of TEN buffer (40 mM Tris–HCl, pH 7.5; 10 mM EDTA; 150 mM NaCl) for 8 min. Jurkat T cells were seeded at 1×10^6 cells per 24 well dish in 400 µl RPMI medium with 10% FCS. They were transfected with lipofectamin™ (BRL), using 8 µl reagent and 2–3 µg DNA per 100 µl reaction and allowing 15 min for micelle formation. The reaction mix was incubated for 5 h with the cells after which 2 ml of fresh medium was added. Cell lysates were obtained by suspending the cell pellet in 100 µl of 0.1 M potassium phosphate buffer (pH 7.3) and freeze/thawing for three cycles. Aliquots of the lysate were assayed for luciferase activity as described (deWet *et al.*, 1987). Transfection efficiency was normalized by assaying for β-galactosidase activity expressed from 0.5 µg of co-transfected RSV-β-gal plasmid (Bonnerot *et al.*, 1987) as described (Herbomel *et al.*, 1984). Each data point shown in the Figures was obtained by averaging duplicate or triplicate samples and is representative for a set of at least two independent experiments. Human USF-1 was expressed from the CMV promoter in pCDNA1 (InVitrogen) and chicken $p54^{ets\text{-}1}$ (Duterque Coquillaud *et al.*, 1988) from an SV–40 promoter in pSG-5 (Green *et al.*, 1988). EtsΔTA encodes amino acids 238 to 441 of chicken $p54^{ets\text{-}1}$ fused to an N-terminal myc tag and USFΔTA codes for amino acids 204–310 of human USF-1 fused to an N-terminal HA tag. Both constructs were expressed from a CMV promoter in RC/CMV (InVitrogen). GAL-USF has been described (Desbarats *et al.*, 1996). For the reporter constructs the –138 to –170 bp region of the HIV-1 LTR was inserted as a multimerized or single copy into the *Xho*I site in front of a tk minimal promoter luciferase reporter (Lim *et al.*, 1992). We used the following oligonucleotides (+ strand):

Wild-type:	5′-TCGACTCATCACGTGGCCCGAGAGCTGCATCC GGAGTAC-3′
Emut:	5′-TCGACTCATC**TGTA**GGCCCGAGAGCTGCATCC GGAGTAC-3′
EBSmut:	5′-TCGACTCATCACGTGGCCCGAGAGCTGC**TAGA** GGAGTAC-3′
Emut/EBSmut:	5′-TCGACTCATC**TGTA**GGCCCGAGAGCTGC**TAGA** GGAGTAC-3′

The luciferase reporter plasmids containing the wild-type form or the –130 to –147 linker scanning mutation of the HIV-1 LTR (Zeichner *et al.*, 1991), and the GAL4 luciferase reporter $17m_2$TATAluc (Desbarats *et al.*, 1996), have been described.

Protein interaction assays in solution

Ets proteins were cloned into the GST fusion vector pGEX-2T (Smith, 1993) using standard protocols. The amino acid junctions of the GST vector with the Ets-1 sequences (in bold type) are: construct 332, GSPH**MKA**; constructs 106/116, GSPH**MGR**; construct 117, GSPHKF**SRG**; construct 118, GSPHMLSGSM**GPI**; construct 119, GSPH**KLS**. GST-PU.1-DBD encodes amino acids 160–264 of human PU.1 with the junction GSPHM**SKK** and GST-Fli-1-DBD encodes amino acids 235–452 of human Fli-1 with the junction GSPHMLSGSPGI**NSG**. GST-Elf-1 encodes full length human Elf-1 in pGEX-3X (Wang *et al.*, 1993). The bacterial expression of GST constructs and the purification of GST fusion proteins were performed as described (Sieweke *et al.*, 1996). *In vitro* translated proteins were generated with a rabbit reticulocyte *in vitro* transcription/translation system (TNT/Promega) using T7 or T3 RNA polymerase and labeling with 100 µCi [^{35}S]methionine/50 µl reticulocyte lysate. Plasmids for *in vitro* transcription of human c-myc, TFE-3, mad, (Gaubatz *et al.*, 1995; Desbarats *et al.*, 1996), E47, E12 (Peverali *et al.*, 1994) and chicken SCL (Goodwin *et al.*, 1992) have been previously described. 5 µl of reticulocyte lysate were diluted to 200 µl with binding buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.05% Triton X100; PMSF/aprotinin/leupeptin protease inhibitor mix) and incubated with 10 µl of GST–protein loaded resin for 1 h at 4°C. After five washes in 1 ml of binding buffer the complexes were dissociated in 20 µl sample buffer (50 mM Tris–HCl, pH 6.8; 2% SDS; 5% β-ME; 10% glycerol; 0.02% Bromophenol Blue) and separated by 12.5% SDS–PAGE (Harlow and Lane, 1988). After staining with Coomassie Blue the gel was enhanced by DMSO/PPO fluorography (Harlow and Lane, 1988) and exposed to X-ray film for 4–12 h.

Protein interaction assay in vertebrate cells

Three 60 mm dishes per sample with 1×10^6 QT6 cells each were co-transfected by the calcium-phosphate method (Graham and van der Eb, 1973) with 2 µg of expression constructs for human USF-1 and 2 µg of a His-tagged, truncated Ets molecule encoding amino acids 333–441 of chicken $p54^{ets\text{-}1}$ (His_6/Ets-DBD). After 48 h the cells were washed with PBS, lysed in 1.5 ml of lysis buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% NP–40) for 20 min and then sonicated six times for 5 s. The lysates were cleared by centrifugation for 10 min at 5000 r.p.m. in an Eppendorf centrifuge and then incubated with 30 µl of Ni^{++} agarose beads (Qiagen) for 1 h on a rotating wheel. After three washes with lysis buffer, complexed proteins were eluted from the beads with 20 µl of sample buffer. All experiments were performed at 4°C in the presence of protease inhibitor mix. Eluted proteins were separated by SDS–PAGE as described for the *in vitro* assays and then transferred to an Immobilon P membrane (Millipore) with a Bio-Rad dry blotter at 2.5 mA/cm^2 using 25 mM Tris–base, 192 mM glycine and 20% v/v Methanol as the transfer buffer. Membranes were blocked in TBS with 4% w/v dry milk overnight and stained with a rabbit antibody against a peptide of human USF-1 corresponding to amino acids 291–310 (Santa Cruz), and a secondary goat anti rabbit/peroxidase antibody (Amersham). Antibody incubations were for 1 h in TBS with 4% w/v dry milk followed by three washes of 15 min in TBS with 0.2% Triton X100. For detection we used the ECL chemiluminescent peroxidase substrate kit from Amersham.

Electrophoretic mobility shift assays (EMSA)

To produce recombinant proteins of the DNA-binding domains of USF-1 and Ets-1 the coding regions for amino acids 194–310 of human USF-1 or amino acids 333–441 of chicken $p54^{ets\text{-}1}$ were inserted into the *Nde*I (USF-1) or *Bam*HI (Ets-1) site, respectively, of a pET 15b vector (Novagen) resulting in the following amino acid junctions with the N-terminal His_6 tag of the vector: USF-DBD, RGSHM**TTR**; Ets-DBD, RGSHMLSGS**MGP**. The proteins were expressed in *E.coli* strain BL21(λDE3)pLysS and purified by affinity chromatography on Ni^{++} agarose beads (Qiagen) under non-denaturing conditions essentially following the supplier's recommendations (Novagen). The binding reaction was performed at room temperature for 15 min in a total volume of 20 µl containing 20 mM Tris, pH 7.5; 80 mM NaCl; 1 mM EDTA; 0.1% TritonX100; 2 mM DTT; 5% glycerol and 5 µg/ml poly dIdC. As a probe we used a silica column-purified (Qiagen), double stranded oligonucleotide corresponding to the –138 to –170 bp region of the HIV-1 LTR and labeled with [γ-^{32}P]dCTP by Klenow fill in (Sambrook *et al.*, 1989) at a concentration of $<$10 pM ($<$10 000 c.p.m. per reaction). For the antibody experiments we used 2 µg of rabbit polyclonal IgG against the C-terminal 20 amino acids of human USF-1 or the C-terminal 20 amino acids of human Ets-1 (both Santa Cruz). All samples were subjected to electrophoresis on a 5% polyacrylamide gel containing 3% glycerol in a 12.5 mM Tris, 95 mM glycine buffer. Band intensity of the autoradiographs was quantified with the NIHimage 1.6 program.

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