Upstream stimulating factor affects human immunodeficiency virus type 1 (HIV-1) long terminal repeat-directed transcription in a cell-specific manner, independently of the HIV-1 subtype and the core-negative regulatory element

Mojgan H. Naghavi,1 Mario C. Estable,3 Stefan Schwartz,1, 2 Robert G. Roeder3 and Anders Vahlne1

1 Division of Clinical Virology, Karolinska Institute, F68, Huddinge University Hospital, S-141 86 Huddinge, Stockholm, Sweden
2 Department of Medical Biochemistry and Microbiology, BMC, Uppsala University, S-751 23 Uppsala, Sweden
3 Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, 1230 York Avenue, New York 10021-6399, USA

Human immunodeficiency virus type 1 (HIV-1) is classified into subtypes on the basis of phylogenetic analysis of sequence differences. Inter- and intra-subtype polymorphism extends throughout the genome, including the long terminal repeat (LTR). In this study, the importance of the upstream stimulating factor (USF)-binding site (E-box) in the core-negative regulatory element (NRE) of the LTR of HIV-1 subtypes A, B, C, D, E and G was investigated. In vivo, USF was found to repress transcription directed from representative HIV-1 LTR sequences of all the subtypes tested in an epithelial cell line, yet activate the same transcription in a T-cell line. Mutation of the core-NRE USF site of the representative subtype B LTR did not affect the cell-specific, subtype-independent, dual role of USF. In vitro binding assays showed that recombinant USF43 interacts with the core-NRE from subtypes B and C, but not A, D, E or G. Thus, USF affects LTR-directed transcription in a cell-specific manner, independently of both the HIV-1 subtype from which the LTR was derived and the core-NRE USF site sequences.

Introduction

Phylogenetic analysis of human immunodeficiency virus type 1 (HIV-1) using mostly the env and gag sequences has led to its classification into at least 11 subtypes (A to K) (Myers et al., 1995; Janssens et al., 1997; Triques et al., 2000) and additional inter-subtype recombinants (Robertson et al., 1995a, b). A significant amount of intra-subtype variability also exists, particularly for the V3 loop of env (Estable et al., 1998b). The long terminal repeat (LTR) has more recently been used for subtyping (Gao et al., 1996; Montano et al., 1997; Naghavi et al., 1999a). As is the case for other regions of the genome, the LTR displays considerable intra-subtype sequence variability (Estable et al., 1996, 1998a).

Taken together, sequence polymorphisms defining subtypes are not neutral. For example, a higher transmission rate has been suggested for Thailand subtype E (Soto-Ramirez et al., 1996). A preference for certain coreceptors has been observed for subtypes C and D (Tscherning et al., 1998; Bjorndal et al., 1999) and subtype A has recently been reported to be less pathogenic (Kanki et al., 1999). In particular, subtype-specific polymorphisms in transcription factor-binding sites within the LTR have the potential to affect the rates of virus replication because the LTR directs transcription of the proviral genome. Indeed, even intra-subtype LTR polymorphism can have a profound effect on transcription directed from the LTR (Estable et al., 1996, 1998a).

The DNA sequences within the prototypical HIV-1 5’LTR can be divided into the negative regulatory element (NRE), the enhancer/promoter region and the sequences encoding a transcriptionally nascent RNA stem–loop structure termed TAR. Although first described as a negative regulatory region, NRE also confers positive regulation and contains binding sites for many cellular transcription factors including upstream stimulating factor (USF), AP1, NFAT, hLEF (reviewed by Gaynor, 1992), E26-specific (Ets) family members (Holzmeister et al., 1993) and RBF-1 and -2 (Bell & Sadowski, 1996; Estable et al., 1996, 1998a, 1999). The enhancer/promoter region...
consists of two highly conserved NFκB sites, three Sp1-binding sites, a TATA box and an initiator element (Gaynor, 1992). The NFκB sites convey activation of transcription in response to a large number of extracellular stimuli such as viral proteins, phorbol esters and multiple cytokines (Gaynor, 1992). However, it is likely that for some of these effects, certainly Ras-responsiveness, other sequences are involved as well (Bell & Sadowski, 1996). The TAR element in conjunction with the viral protein Tat confers critical regulation of transcription elongation. The Tat–TAR interaction in an apparent ternary complex with P-TEFb results in the phosphorylation of the Pol II CTD by the CDK9 component of P-TEFb (reviewed by Karn, 1999).

Subtype-specific differences in the HIV-1 LTR of subtypes E and C have been associated with a higher promoter/enhancer activity as well as a faster spread of these viruses in southeast Asia (Thailand, HIV-1 subtype E) and sub-Saharan Africa (Zimbabwe, HIV-1 subtype C) (Montano et al., 1997, 1998). It has also been suggested that the conversion of an NFκB site, present in Thai HIV-1 subtype E, into an Ets transcription factor family member protein (GABP)-binding site may result in enhanced Tat trans-activation (Verhoeef et al., 1999). We have recently reported that subtype C promoter/enhancer activity was, on average, higher than the activities of the other subtypes and this correlated with the presence of a third potential NFκB site as well as an alternative core-NRE (position −174 to −163) (Naghavi et al., 1999a, b). The latter site contains a characteristic E-box motif, CAC(A/G)TG, which has been shown to be a target for USF (Sawadogo & Roeder, 1985a, b; Gregor et al., 1990). USF is a member of the basic helix-loop-helix/leucine zipper family of transcription factors and is constitutively expressed in many tissues (Chodosh et al., 1986; Sawadogo et al., 1988). USF was initially characterized as a positive activator of major late promoter transcription of adenovirus type 2 (Carthew et al., 1986; Sawadogo & Roeder, 1985b). In the context of HIV-1, several studies have demonstrated both a negative (Lu et al., 1989, 1990; Giacca et al., 1992) and a positive (Maekawa et al., 1991; Zeichner et al., 1991; Moses et al., 1994; di Fagagna et al., 1995; Sieweke et al., 1998) effect of USF on HIV-1 subtype B LTR-directed transcription. Despite this, the impact of USF on LTR-directed transcription from different subtypes is unknown. However, it has been shown that the USF site in the core-NRE deviates from the E-box consensus in the majority of naturally occurring subtype B isolates (Estable et al., 1996). This suggests that the effect of USF upon LTR-directed transcription will vary depending on the isolate or prototypical subtype sequences in the core-NRE USF site.

In this study, we have further investigated the functional importance of the core-NRE site from LTRs of HIV-1 subtypes A to E and G, both in T-cells and in epithelial cells, with respect to USF. Our results show that USF activates LTR-directed transcription in T-cells, while it functions as a transcription repressor of the HIV-1 LTR in epithelial cells. This dual tissue-specific effect of USF was unexpectedly subtype-independent despite variability within the core-NRE USF-binding site. The fact that the effects of USF are independent of the core-NRE USF site was corroborated by electrophoretic mobility shift assays (EMSAs) showing that USF binds strongly to subtype B, weakly to subtype C, but does not bind to the core-NRE of subtypes A, D, E or G. Taken together, our data show that USF affects HIV-1 LTR-directed transcription from all the subtypes tested and suggests that USF is acting through an unidentified element conserved in subtypes A to E and G.

**Methods**

**Plasmid constructs.** The LTRs used in this study were previously described by Naghavi et al. (1999a, b) and each of the respective constructs described drive the expression of CAT. The GenBank accession numbers of the sequences used are as follows: AF102, AF199, AF202–AF204, AF208, AF212, AF216, AF219, AF220, AF223. The plasmids pNLC4-3 (Adachi et al., 1986), pNLCATB (Tan et al., 1995), pNLNCATXSXB (Naghavi et al., 1999b), pCMV-Tat (Sokolowski et al., 1998) and pCMVHGH (Ramirez-Solis et al., 1990) have been described previously. pCMVUSF drives the expression of USF from the CMV promoter within the pcDNA mammalian expression vector (Invitrogen) and pFlagUSFpET-11d drives expression of FLAG-tagged USF from the pET11d bacterial expression vector (Novagen). To generate pCMVAUSF, pCMVUSF was digested with NcoI and Xhol, blunt-ended with Klenow and ligated.

**Mutant LTR.** To generate HIV-1 LTRs containing core-NRE deletions, pCR2-1 (Invitrogen) containing the LTR from subtype B (TA-SF2-1) was used as a template (Naghavi et al., 1999a). PCR was performed in two separate reactions containing 1 µM each of oligonucleotides LTR1-SX and NRE-1 (mix I) or LTR3-SB and NRE-2 (mix II). As previously described (Naghavi et al., 1999b), LTR1-SX contains StuI and XhoI cloning sites and LTR3-SB contains ScaI and BanHI cloning sites. NRE-1 and NRE-2 primers are complementary and each contain a 12 bp deletion which removes the core-NRE (position −174 to −163). The sequences of these primers are NRE-1, 5′ GCCCTCGGGGCTGC- TAGAGG 3′ and NRE-2, 5′ CCTCTTAGCCGAGACGAC 3′. Equal amounts of the two PCR fragments generated were mixed and used as template in a nested PCR reaction primed by the addition of oligonucleotides LTR1-SX and LTR3-SB as previously described (Naghavi et al., 1999b). The XhoI–BanHI PCR fragment was then transferred into pNLCATXSXB (Naghavi et al., 1999b).

To generate a full-length HIV-1 clone with a deletion of the core-NRE, the resulting mutant LTR sequence was inserted as a XhoI–BanHI fragment into pNL4-3 as previously described (Naghavi et al., 1999b). The sequence of the core-NRE mutant (mSF2-1) was confirmed by sequencing.

**Cells and transfections.** The human T-cell lymphoma line Jurkat (ATCC) was seeded at 1.5 x 10⁶ cells per well in a 12-well dish. The next day, the cells were transfected using 2 µl FuGENE 6 transfection reagent (Boehringer Mannheim) per µg DNA. Transfection efficiency was normalized to human growth hormone (hGH) expressed from the cotransfected pCMVHGH plasmid, which was present in all transfections as described previously (Naghavi et al., 1999b). A titration of various pCMVUSF plasmid concentrations (0–7 µg) against the pNLNCATB plasmid was used as a control for the effect of USF at different concentrations. Each of the pNLNCAT-derived plasmids (in 3 µl aliquots) were mixed with 1 µg pCMVHGH in the presence or absence of 1 µg
USF regulation of HIV-1 transcription

pCMVtat and/or 7 µg pCMVUSF or pCMVAUSF. The total amount of DNA in each transfection was adjusted to 12 µg by the addition of pBluescript DNA. The FuGENE transfection reagent was added into the DNA mix according to the standard protocol (Boehringer Mannheim). At 48 h post-transfection, cell lysates were obtained by suspending the cell pellets in 500 µl of lysis buffer (100 mM Tris, pH 7.6, and 1% Triton X-100), freeze–thawed three times and centrifuged at 15 000 g for 15 min at 4 °C. CAT and hGH activity was measured as described below.

Transfection of HeLa cells was performed with the DNA–calcium phosphate coprecipitation technique as described previously (Naghavi et al., 1999b). DNA was mixed exactly as for transfection of Jurkat cells except that the total amount of DNA was adjusted to 15 µg by the addition of pBluescript DNA. Cells were harvested and analysed 24 h post-transfection as described above. In order to analyse the effect of USF on the wt and core-NRE-deleted LTR from subtype B (TA-SF2-1), the respective pNL4-3-derived plasmid was included in the transfection of HeLa cells. The effect of USF at various concentrations was tested against pNL4-3:1B as described for pNLCATB. Cell culture supernatants were collected at various times post-transfection (0, 24, 48, 72 and 92 h) and the production of hGH and HIV-1 p24ag was measured as described below.

ESMs were performed as previously described (Estable et al., 1999). Nuclear extracts were prepared from HeLa cells and the human T-cell lymphoma line HUT-78 as previously described (Dignam et al., 1983). Oligonucleotides containing consensus core-NRE for different LTR subtypes of HIV-1 (NRE-

<table>
<thead>
<tr>
<th>Nucleotide Sequence</th>
<th>CAT Activity</th>
<th>hGH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 79-106</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 174-193</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 194-213</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 214-233</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 234-253</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 254-273</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 274-293</td>
<td>CAT</td>
<td>hGH</td>
</tr>
<tr>
<td>Position 294-313</td>
<td>CAT</td>
<td>hGH</td>
</tr>
</tbody>
</table>

Fig. 1. Nucleotide sequence alignments of HIV-1 LTRs are shown. The core-NRE (position −174 to −163) and the enhancer region (position −106 to −79) taken from the complete sequence alignment (position −382 to +113) are shown. The letters before the sample code denote the country of origin of the virus strain as follows: UG, Uganda; SE, Sweden; ET, Ethiopia; GM, Gambia; SO, Somalia; TH, Thailand. The letter after the sample code indicates the HIV-1 subtype (subtypes A to E and G) based on the LTR region. Deviations from the subtype B consensus are indicated.

Results

USF represses LTR-directed transcription in HeLa cells, independently of the subtype from which the LTR was derived

The relevant part (position −174 to −79) of the LTR alignment is presented in Fig. 1. To test the effect of USF on HIV-1 LTR-directed transcription in the context of a reporter gene, five of the previously described CAT constructs each containing LTR sequences from subtypes B to E and G (SE5611:3B, GM6439:1C, UG5609:1D, TH6098:1E and GM6139:1G) (Naghavi et al., 1999b) were analysed in transient transfections in HeLa cells. We first tested the effect of USF on the reference plasmid pNLCATB (Tan et al., 1995). From 10 independent transfection experiments, ectopic over-expression of USF inhibited transcription from the pNLCATB HIV-1 LTR by nearly 3-fold regardless of the presence of Tat (Fig. 2a, b). This inhibitory effect of USF was dose-dependent, with a maximum inhibition at ≥ 7 µg of the pCMVUSF plasmid (Fig. 2e). To verify that the observed inhibitory effect was induced by the ectopic production of USF, pCMVΔUSF, in which the USF ORF was deleted, was included in some of the cotransfections. No inhibition of LTR-mediated transcription was observed for pNLCATB in the presence of 7 µg pCMVΔUSF in HeLa cells (Fig. 2f). The same experiment was performed with LTRs from different HIV-1 subtypes. Interestingly, the pattern of USF inhibition of LTR-directed transcription from different subtypes was similar in the absence (Fig. 2a) or presence (Fig. 2b) of Tat. Subtype C LTR (GM6439:1C) was consistently more active, followed by a wide range of transcriptional activities for the LTRs from the different subtypes, with the subtype D LTR displaying the
**Fig. 2.** USF effects on LTR-mediated transcription in HeLa cells. CAT activity of the various HIV-1 LTRs from different subtypes in the absence (a) or presence (b) of Tat or/and ectopic USF43. Cell extracts from untransfected HeLa cells (HeLa-EXT) were used as a negative control and the reference plasmid pNL CATB acted as a positive control. CAT refers to arbitrary CAT units from the CAT ELISA. Error bars indicate SD values. Mean p24 gag activity of different full-length HIV-1 genomes in the absence
USF regulation of HIV-1 transcription

USF represses LTR-directed transcription in HeLa cells, independently of the USF-binding site sequences in the core-NRE

In order to test the functional importance of the USF-binding site in the NRE, a 12 bp deletion, which included the USF site, was introduced into the NRE of the LTR sequences from subtype B (see Fig. 6b). The wt (SF2:1B) or mutant (mSF2:1B) LTR clones were then transferred into pNLCatXSXB or pNL4-3 plasmids (see Methods) and tested in transient transfections in the absence or presence of the viral protein Tat or/and USF in HeLa cells. The levels of CAT and p24<sup> gag </sup> production from the different LTRs was in agreement with the CAT activity observed with the same subtype LTRs. Strikingly, in the presence of USF, we observed up to a 17-fold inhibition of p24<sup> gag </sup> production from the different viruses (Fig. 2d). Thus, USF had an inhibitory effect on the various LTR sequences from the different subtypes and also in the context of the complete viral genome.

lowest activity. In total, we observed 1.5- to 4-fold inhibition of HIV-1 LTR-directed transcription for all of the studied clones (except for the low LTR activity clone UG5609:1D) in the presence of USF, regardless of the presence of Tat. This suggests that the negative effect of USF was independent of the sequence variations in the USF-binding site in the different HIV-1 subtypes (see below).

To investigate whether USF transcription repressive effects translate into a repression of transcription in the context of the complete viral genome, we introduced seven of the LTR sequences (UG5218:1A, SE5611:3B, ET2222:1C, UG5609:1D, TH6098:1E, GM6139:1G and GM6150:2G) of different subtypes (A to E and G) into the 5'LTR position in the infectious HIV-1 molecular clone pNL4-3 (Adachi et al., 1986). The clones were transiently transfected, in the absence or presence of pCMVUSF, into HeLa cells in triplicate samples in two independent experiments. The extracellular p24<sup> gag </sup> levels were then determined at various times post-transfection. Mean p24<sup> gag </sup> activity for the various infectious clones in the absence of USF is shown in Fig. 2(c). The highest p24 activity was observed for viruses from subtype C (ET2222:1C), E (TH6098:1E) and G (GM6139:1G and GM6150:2G) in accordance with previously published results (Naghavi et al., 1999b). Subtype B (SE5611:3B) showed an intermediate level of p24<sup> gag </sup> production, while subtypes A (UG5218:1A) and D (UG5609:1D) showed the lowest LTR promoter activity. This pattern of p24<sup> gag </sup> production of the clones with various LTRs was in agreement with the CAT activity observed with the same subtype LTRs. Strikingly, in the presence of USF, we observed up to a 17-fold inhibition of p24<sup> gag </sup> production from the different viruses (Fig. 2d). Thus, USF had an inhibitory effect on the various LTR sequences from the different subtypes and also in the context of the complete viral genome.

(c) or presence (d) of excess USF in cotransfected HeLa cells. The extracellular p24<sup> gag </sup> levels were determined at various times post-transfection (0, 12, 24, 48 and 72 h). Supernatant from transfected HeLa cells (HeLa-sup) and the infectious clone pNL4-3 was used as negative and positive controls, respectively. (e) CAT activity of pNLCatB (HIV-1 LTR from subtype B) in the presence of the empty USF expression vector pCMVΔUSF, indicated as dUSF.
Fig. 4. Mean p24(\textsuperscript{pg/ml}) activity of the wt or core-NRE-deleted LTRs from HIV-1 subtype B in the absence or presence of Tat or/and ectopic USF\textsuperscript{43} in cotransfected HeLa cells. p24(\textsuperscript{pg/ml}) concentrations were measured at various times post-transfection (0, 24, 48, 72 and 92 h). Supernatant from untransfected HeLa cells (HeLa-sup) was used as a negative control. p24(\textsuperscript{pg/ml}) activity of the wt (pNL4-3:1B) or the core-NRE-deleted (mpNL4-3:1B) infectious clones \(\text{(a)}\) in the presence of ectopic USF\textsuperscript{43} \(\text{(b)}\), Tat \(\text{(c)}\), and Tat + USF\textsuperscript{43} \(\text{(d)}\), Tat + dUSF \(\text{(e)}\), and Tat + USF \(\text{(f)}\).
Notably, the fold inhibition of USF was similar for the wt and mutant clones both in the absence (Fig. 3a) and in the presence (Fig. 3b) of Tat (2-2.5-fold and 1-4- to 1.6-fold, respectively). Importantly, this indicates that the core-NRE site is not of functional importance for the inhibitory effect of USF. The levels of hGH activity produced from pCMVHGH, which is driven by the CMV promoter, were similar both in the absence or in the presence of USF (Fig. 3c), demonstrating that USF had no effect on the CMV promoter.

In addition, we investigated the effect of USF on the same wt and core-NRE mutant LTRs in the context of the complete viral genome. Mean p24\textsubscript{gag} activity for the wt and mutant viruses is shown in Fig. 4. The p24\textsubscript{gag} levels were similar for the wt (pNL4-3 : 1B) and mutant (mpNL4-3 : 1B) viruses in the absence of Tat (Fig. 4a). In this experiment the wt virus showed a lower p24\textsubscript{gag} activity (1-8-fold) than that of the mutant virus at 92 h post-transfection in the presence of Tat (Fig. 4c). However, one of the triplicates in this experiment gave a p24\textsubscript{gag} value of more than 13-fold less than the other two thus reducing the mean by 367 for pNL4-3 : 1B. In another independent experiment, no such differences between the wt and mutant viruses were observed. Also, cotransfections with the USF plasmid resulted in an inhibition of virus transcription for both of the tested viruses (Fig. 4b, d) regardless of the presence of Tat. Again, no inhibition of the LTR-mediated transcription was observed for either pNL4-3 : 1B or mpNL4-3 : 1B viruses in the presence of pCMV\textsubscript{ΔUSF} in HeLa cells (Fig. 4e). The repressive effect of USF was dose-dependent with a maximum inhibition at \( \geq 7 \mu\text{g} \) of the pCMVUSF plasmid as observed in the CAT assay (Fig. 4f). These findings further corroborate the theory that ectopic expression of USF is responsible for the repressive effects we report and that these effects are independent of the core-NRE USF-binding site sequences.
M. H. Naghavi and others

Fig. 6. (a) DNA gel-shift analysis of the core-NRE in the various HIV-1 LTRs from subtypes A to E and G. The consensus core-NRE from subtype B LTR was used as radiolabelled probe (NRE-B). Competition assays were performed with cold competitors representing the consensus core-NRE sequences from subtypes A to E and G or non-consensus core-NRE sequence from a subtype C strain, NRE-5710. Lane 1 indicates the free probe. The binding of the probe to the nuclear extract from HeLa cells (HeLa-NE) is shown in lane 2. The competition assays were performed in the presence of a 100-fold molar excess of the cold competitors in the presence of HeLa-NE (lanes 3–9) or a recombinant USF43 protein (rUSF43) (lanes 10–15). NS, non-specific complex. (b) Synthetic oligonucleotides used for DNA gel-shift analysis. The oligonucleotides represent the consensus core-NRE sequences in the LTR of subtypes A to E and G (NRE-A to -E and -G) or non-consensus core-NRE sequence from a subtype C strain (NRE-5710). The core-NRE USF-binding site (E-box) is indicated.

USF activates LTR-mediated transcription in Jurkat cells, independently of the subtype from which the LTR was derived

To investigate whether USF also functions as a down-regulator of LTR-mediated transcription in T-cells, six of the previously described CAT clones containing LTR sequences from subtypes A to E and G (UG5218:1A, pNLACTB:B, GM6439:1C, UG6357:1D, TH6098:1E and GM6139:1G, respectively) (Naghavi et al., 1999b) were analysed in transient transfections in T-cells. Jurkat cells were cotransfected in triplicate with or without USF in the presence of Tat as
described for HeLa cells (Naghavi et al., 1999b). Surprisingly, USF caused an upregulation of transcription from the HIV-1 LTR in Jurkat cells (Fig. 5a). The USF activation in Jurkat cells was between 1.7- and 3.2-fold. The stimulatory effect of USF was dose-dependent, with a maximum stimulation obtained with pCMVUSF at 2 µg and above (Fig. 5c). A similar pattern of CAT activity was observed in the absence or presence of USF in HUT-78 cells (data not shown). Thus, USF activates LTR-directed transcription in T-cells, independently of the subtype from which the LTR was derived.

**USF activates LTR-directed transcription in Jurkat cells, independently of USF-binding site sequences in the core-NRE**

We also tested the functional importance of the USF-binding site in Jurkat cells in cotransfection experiments with the wt (SF2:1B) and mutant (mSF2:1B) LTR clones as described above. As shown in Fig. 5(b), the core-NRE mutant clones showed a lower CAT activity (1.7- to 1.9-fold) than the wt clones, both in the absence and in the presence of USF, suggesting that this region is required for efficient transcription in T-cells. The USF activation was 1.6-fold and similar for the wt and the mutant-NRE clones. Again, no activation of the LTR-mediated transcription was observed for the wt or the mutant clones in the presence of pCMV∆USF in Jurkat cells (Fig. 5d). These findings confirmed the observations from transient transfections of HeLa cells, which showed that the core-NRE site is not the target of USF. Taken together, USF upregulates LTR-mediated transcription in Jurkat cells and this positive effect of USF is independent of the core-NRE.

**USF interacts in vitro with the core-NRE USF-binding sites from only a subset of HIV subtypes**

As shown in Fig. 6(a), two complexes form between proteins in HeLa nuclear extracts and a radiolabelled double-stranded oligonucleotide comprising sequences from the USF core-NRE site of the HIV-1 LTR subtype B consensus (lane 2) that contains a perfect USF E-box site (CACRTG). Whereas the lower band was shown to be non-specific by using an unrelated probe (results not shown), the upper complex (labelled USF) was specific to the core-NRE USF site B, since it is abolished with 100-fold competition with unlabelled core-NRE USF site B (lane 4) or C (lane 5), but not with core-NRE USF sites from the other subtypes (lanes 3, 6–9). That this band represents USF interaction is strongly suggested by the sequences encompassed within the sites that did not compete, since they do not conform to the consensus USF target sequence, CACRTG. Further evidence that the major band represented USF interaction could be deduced from EMSAs with rUSF43 (lanes 10–15), where a single band at the same position as the band from HeLa nuclear extract behaves in an identical manner when competed with the various subtype core-NRE consensus sequences. Furthermore, a complex between rUSF43 and radiolabelled core-NRE consensus sequences was not detected for any subtype other than B and C (Fig. 7). Lanes 3–8 demonstrate that rUSF43 could only interact with the core-NRE from subtype B and to a lesser extent with that from
Discussion

In both HeLa and Jurkat cells, the different subtype LTRs that we have examined directed a wide range of transcriptional activity, as has been reported for intra-subtype variants (Estable et al., 1996) as well as for inter-subtype variants (Naghavi et al., 1999b). This highlights the apparent fragility of the LTR to mutations affecting transcription potential. For example, the subtype D LTR (UG5609:1D), with the lowest directed activity, was previously shown to contain mutations in critical transcription factor-binding sites (Naghavi et al., 1999b). Similarly, the higher activity from subtype C correlates with the presence of a third potential NFkB site and an alternative core-NRE (Naghavi et al., 1999b). The importance of these subtype C-specific mutations for the higher transcriptional activity of these viruses is under investigation.

Our data indicate that USF functions as a repressor of HIV-1 LTR-directed transcription in HeLa cells. This result agrees with previous findings that suggested an inhibitory effect of USF in HeLa as well as in COS-1 cells (Giacca et al., 1992). However, two in vitro transcription studies performed with nuclear extract from HeLa cells suggest that USF is a positive regulator of LTR-mediated transcription (Maekawa et al., 1991; di Fagagna et al., 1995). The apparent discrepancy is likely to be a result of the inherent differences when examining transcription in vivo versus in vitro, e.g., nuclear extracts may not necessarily re-capitulate the in vivo conditions. One possibility would be that the in vitro system lacks a partner for USF that is present in vivo and this confers repression when partnered with USF. Another explanation for the discrepant results would be the usage of shorter LTR regions in these studies, e.g., lacking the initiator element and the TAR sequences (di Fagagna et al., 1995) or AP1- and NFAT-binding sites (Maekawa et al., 1991). The ability of USF to repress LTR-directed transcription was independent of subtype and, moreover, independent of the core-NRE USF-binding site. This suggested that USF is acting through a site other than the core-NRE E-box. Our in vitro EMSA data further substantiate this conclusion, since only subtype B and C consensus sequences interacted with USF. Importantly, the repressive effects of USF were evident in the presence of Tat and more pronounced in the context of whole virus. The important distinction is that Tat is expressed either under the control of the CMV promoter (transient transfections) or under the control of the respective HIV-1 LTRs (whole genome context). USF would lengthen the time for the first Tat to be made and the effects appear more pronounced in the context of whole virus, just as we have observed.

We have shown that USF activates LTR-directed transcription in T-cells, in contrast to the repression in HeLa cells. This finding confirms earlier studies that indicate a positive effect of USF in T-cells (Sieweke et al., 1998) and macrophages (Moses et al., 1994). Here we extend these previous findings to show that this activation, as is the case for the repression in HeLa cells, is subtype-independent. As was the case for the repressive effects in HeLa cells, we have further shown by mutagenesis of the core-NRE and through in vitro EMSAs that these effects are not mediated by the core-NRE USF-binding site and occur in the presence or absence of Tat. Additional experiments for demonstrating how USF is recruited to the LTR as well as the role of overexpressed USF in primary cells are under investigation.

The apparent dual, tissue-specific, repressor/activator role for USF that we detect takes on physiological importance because it is subtype-independent. Unfortunately, a subtype-independent role for most transcription factors binding to putative elements within the prototypical HIV-1 LTR has not been investigated. However, sequence analysis of variants indicates that very few elements within the HIV-1 LTRs naturally occurring in patients adhere to the prototypical sequence (Estable et al., 1996). USF may therefore play a role in the control of HIV-1 latency versus activation of all HIV-1 variants. Although we have not determined the mechanism by which USF could achieve this role, the interaction of USF with another cellular transcription factor is possible since USF is a protein capable of dimerization. Direct interaction between USF and Ets-1 has recently been suggested to be required for full transcriptional activity of the HIV-1 LTR in T-cells (Sieweke et al., 1998). It has been speculated that LEF, Ets-1 and USF function together in a complex that maintains the promoter in an open configuration (Jones & Peterlin, 1994). In addition, the activation of the LTR by NF-IL6 has been suggested to be mediated through a direct interaction of NF-IL6 with other LTR-binding proteins rather than an interaction of NF-IL6 with its binding site present also in the HIV-1 core-NRE (Tesmer et al., 1993). Importantly, USF may bind to another unrelated binding site in the initiator element (Inr) in the HIV-1 LTR (Du et al., 1993). Cooperative interaction of transcription initiation factor TFII-I and USF at both Inr and E-box sites has been suggested (Meisterernst et al., 1991; Roy et al., 1991, 1997; Chiang & Roeder, 1995). An additional consideration is that USF may affect HIV-1 LTR-directed transcription through interactions with E-boxes present in cellular genes. In this regard, sequence similarity between core-NREs of HIV-1, IFNγ and IL-2Rα has been reported (Nourbakhsh et al., 1993; Smith & Greene, 1989).

More recently, USF has been suggested to be involved in the pathogenesis of AIDS (Simm et al., 1996; Rousseau et al., 1997; Moriuchi et al., 1999). It has also been suggested that
USF plays an important role both in virus expression and in virus entry through upregulation of the promoter activity of CXCR4 (Moriuchi et al., 1999). On the basis of both these findings and our data, USF appears to have at least the potential to affect HIV-1 replication both positively and negatively.

Data about HIV-1 biological activity, transmission and pathogenicity are predominantly based on subtype B virus analysis. Although these viruses are prevalent in Western countries, Asia and Africa, they account for only a small number of HIV-1 infections worldwide. However, subtype C accounts for approximately half of all HIV-1 infections in the world (WHO, 1998). Next to subtype C, subtype E is the most prevalent in the world (Burke, 1996). This faster expansion and the global dominance of subtype C and E viruses have been correlated with a higher transcriptional activity of these subtypes (Montano et al., 1997, 1998). However, the faster spread of these viruses may just be a consequence of the virus epidemiology in highly populated areas such as India, sub-Saharan Africa (HIV-1 subtype C) and Thailand (HIV-1 subtype E). We have also observed a higher transcriptional activity of subtype C viruses (Naghavi et al., 1999b). In addition, inter-subtype recombination within the 5′LTR of subtypes A, C and D has been suggested to lead to increased fitness of viruses (Blackard et al., 1999). A recent study suggests that although minor sequence alterations in the LTR of subtype E viruses result in subtle differences in LTR function, they can have a significant impact on virus replication (Jeeninga et al., 2000). Therefore, further investigation of the divergent transcriptional regulation in relation to pathogenesis and transmission of non-subtype B viruses is important. Further understanding of such diversity may provide us with preventive and therapeutic strategies against AIDS.

We are grateful to Dr G. J. Goodall (Department of Microbiology and Immunology, University of Adelaide, Australia) for kindly providing us with the pCMVHGH plasmid. We also thank Dr David Markowitz (Department of Internal Medicine, The University of Michigan, Ann Arbor, Michigan, USA) for critical reading of the manuscript. This work was supported by the Swedish Medical Research Council and by grants from the Karolinska Institute (no. K2000-06X-09501-10B). M.C.E. is a fellow of the Canadian Medical Research Council.

References


M. H. Naghavi and others


Smith, R. M. & Greene, W. C. (1989). The same 50 kDa cellular protein binds to the negative regulatory elements of the interleukin 2 receptor.
alpha-chain gene and the human immunodeficiency virus type 1 long terminal repeat. *Proceedings of the National Academy of Sciences, USA* 86, 8526–8530.


Received 13 July 2000; Accepted 22 November 2000