FEBS Letters 582 (2008) 3903–3908

Specific interaction of TFII-I with an upstream element on the HIV-1 LTR regulates induction of latent provirus

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Received 25 April 2008; revised 19 September 2008; accepted 16 October 2008

Available online 29 October 2008

Edited by Aleksander Benjak

Abstract RBF-2 is a factor comprised of a USF1/2 heterodimer, whose association with a highly conserved upstream element (RBEIII) on the HIV-1 LTR requires a co-factor TFII-I. We have identified specific nucleotides, immediately 3′ of RBEIII that are required for stable association of TFII-I with this region of the LTR. Mutations that inhibit interaction of TFII-I with DNA also prevent stimulation of USF binding to RBEIII, and render the integrated LTR unresponsive to T cell signaling. These results demonstrate an essential role of TFII-I bound at an upstream LTR element for viral replication.

Keywords: HIV-1; TFII-I; USF; RBF-2; MAPK signaling

1. Introduction

Transcription from the HIV-1 long terminal repeat (LTR) is tightly linked to T-cell activation through the function of factors responsive to signaling by the T-cell receptor and cytokines, including NF-κB, NFAT, GABP/Ets and API bound to the LTR enhancer region (reviewed in Ref. [1]) (see Fig. 1A). Additionally, we have previously shown that RBF-2, comprised of USF1, USF2 and TFII-I, bound to a highly conserved upstream element (RBEIII, Fig. 1A) is essential for induction of the LTR in response to MAPK signaling in T cells [2,3]. RBEIII is an atypical binding site for USF, but is as highly conserved on LTRs from patients as are the TATA box and NFAT/NF-κB enhancer elements ([2,4] and see Table 1). In contrast, the prototypical HIV-1 LTR bears a strong consensus E box element for USF further upstream that is less well conserved (Fig. 1A and Table 1). USF binds to RBEIII with several hundredfold less affinity than a consensus E box in vitro, but this interaction is strongly stimulated by addition of TFII-I, without formation of a stable ternary complex [2]. Mutation of the core RBEIII element prevents induction of the integrated LTR by α-CD3 TCR crosslinking, and co-treatment with PMA and ionomcycin, which activate the Ras-MAPK and calcinuerin-NFAT pathways, respectively [2,3]. Furthermore, integrated LTRs with mutations in RBEIII consistently display elevated basal transcription relative to wild-type [2], and concomitantly have reduced levels of associated HDAC3 [3]. We have proposed that USF 1/2 and TFII-I bound at RBEIII are involved in repression of the latent provirus, through recruitment of HDACs, but are also necessary for enabling induction of the LTR in response to MAPK signaling, perhaps by maintaining organization of the LTR in a conformation amenable to activation by MAPK-responsive transcription factors bound to the enhancer region [1].

Although we have observed TFII-I from Jurkat nuclear extracts bound to RBEIII-containing oligonucleotides in EMSA [3], and have shown that TFII-I co-purifies with USF1 and USF2 using RBEIII element-specific affinity chromatography [5], a specific interaction between TFII-I and upstream sequences on the HIV-1 LTR has not been identified. In this report, we define nucleotides required for direct interaction of TFII-I with this region of the LTR, immediately 3′ flanking the RBEIII core USF1/2 binding site, and show that this specific interaction is necessary for induction of latent provirus.

2. Materials and methods

2.1. Recombinant DNA molecules

Oligonucleotides for construction of plasmids, mutagenesis, DNaseI footprinting and Chromatin Immunoprecipitation are detailed in Supplementary Table 1. The pTYeGFP-WT reporter virus construct was produced by replacing the enhancer-less 3′ LTR in pTYeGFP [6] (obtained from the NIH-AIDS Reagent Program) with an LTR fragment amplified from pLAI using primers oTM237 and oTM238 containing unique EcoRI and KpnI restriction sites. LTR mutations were created by site directed mutagenesis in pTM3235, which is pBluescript containing the wild-type EcoRI/KpnI LAI LTR fragment.

2.2. Cell culture and viral replication assays

Lymphoblastoid Jurkat T-cells, and Jurkat-Tat T-cells were obtained from the NIH AIDS Reagent Program. Human Kidney 293T, and Sf21 insect cells were obtained from the American Type Culture Collection (ATCC). Jurkat T-cells were grown in 1640 RPMI + 10% Fetal Bovine Serum (FBS) supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml), and maintained at 37 °C in a 5% CO_2 atmosphere. The Jurkat-Tat, and derivative lines with integrated reporter virus, were maintained under the same conditions, except that 400 μg/ml G-418 was added to the media. Human kidney 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS (Gibco-BRL) supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml), and maintained at 37 °C with a 5% CO_2 atmosphere. Sf21 insect cells were grown in TC-100 insect media + 10% FBS (Gibco-BRL) and maintained at 27 °C.

The pTYeGFP-LTR reporter viruses were produced by co-transfection of 293T cells with Gag/Pol, Rev, Tat, and VSV-G expression plasmids as described [6]. Infected Jurkat-Tat cells were monitored for eGFP expression 24 h post-transfection and sorted into 96 well plates for isolation of individual clones. Cells with integrated reporter virus were cultured in 75 cm^2 culture flasks to a cell density of $\sim 2.0 \times 10^5$/ml prior to induction with PMA (25 ng/ml) and ionomycin (1 mM) for 24 h.

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0014-5793/$34.00
doi:10.1016/j.febslet.2008.10.032

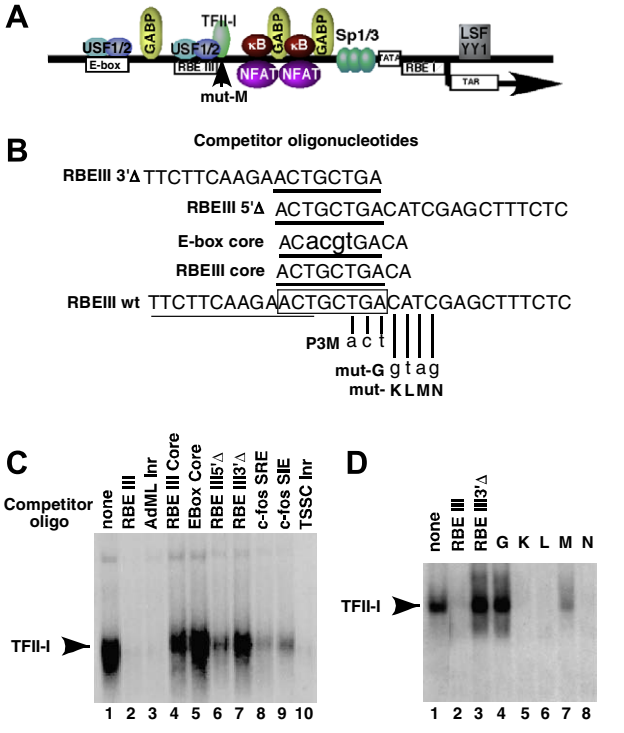

Fig. 1. *Panel A*. Schematic representation of the chromosomally integrated HIV-1 LTR, indicating positions of binding sites for USF1/2, GABP, NFAT/NF-κB, Spl, TFII-I and LSF/YY1. *Panel B*. Sequences of oligonucleotides used as competitors in panels C and D. The RBEIII core is boxed and the location of a sequence resembling an Inr [14] underlined. Nucleotide substitutions are indicated in lower case. *Panels C and D*. EMSA was performed with recombinant TFII-I and the RBEIII wt oligonucleotide probe (Panel B). Unlabeled competitor oligos, as indicated above, were added in 100-fold molar excess.

Table 1
Conservation of elements on the HIV-1 LTR.

Factor	Consensus	Location[a]	Frequency[b]
TBP	TATAA	−28	81
Spl	GGGCGG	−55, −65, −79	74
NFκB	GGGACTTTCC	−90, −104	99
USF/RBEIII	ACTGCTGA	−129	93
TFII-I	CTGA**CATC**	−125	99.5
USF	CACGTG	−166	30

[a]Location of the element(s) relative to the transcriptional start.
[b]Proportion (%) of full HIV-1 subtype B genome sequences in the HIV sequence database with an exact match to the indicated consensus sequence.

2.3. Protein–DNA interaction assays

USF1, USF2 and TFII-I were produced in Sf21 insect cells using baculovirus [2]. Jurkat nuclear extracts were prepared as described previously [7]. Oligonucleotide probes for use in EMSA (Supplementary Table 2) were labeled by end filling reactions with Klenow and unincorporated label was removed using Sephadex G-50 spin columns (Pharmacia) [2]. EMSA binding reactions were performed as previously described [2,3], and resolved on non-denaturing polyacrylamide gels for 5 h at 20 V/cm. Footprinting templates were produced from wild-type or mutant LAI LTRs by amplification using primers oTM122 and oTM123. The fragments were digested with AscI and labeled by end filling with Klenow. Unincorporated label was removed, and the probes digested with XbaI and gel purified to minimize signal from non-specific end-labeling. DNaseI footprinting reactions were performed as previously described [8] Chromatin immunoprecipitation assays with USF1, USF2 and TFII-I antibodies were as in previous experiments [2,3].

3. Results

3.1. Nucleotides immediately 3′ of the RBEIII are required for interaction with TFII-I

TFII-I was initially identified as a protein bound to the AdML initiator element (Inr) [9], and was subsequently observed bound to various upstream promoter sequences in association with additional factors [10–13]. However, there is some controversy as to whether TFII-I recognizes a specific sequence consensus [9,14], and it was even suggested that TFII-I family members might be capable of binding different specific sequences through the multiple I-repeats [15]. We found that recombinant TFII-I was able to form a complex on its own with an RBEIII-containing oligonucleotide in EMSA (Fig. 1C and lane 1), and this interaction was strongly competed by excess unlabeled WT RBEIII oligo (lane 2) and oligos bearing the AdML Inr (lane 3) or *TSSC* Inr elements [16] (lane 10). Oligonucleotides bearing the *c-Fos* serum response element (SRE) and serum inducible element (SIE), previously shown to bind TFII-I [12], also competed with the RBEIII oligonucleotide, although somewhat less efficiently (Fig. 1C, lanes 8 and 9). These results indicate that interaction of TFII-I near RBEIII may involve a DNA binding function for which a consensus has been proposed [9,14]. In examining this region of the LTR we noticed that 11 of 13 nucleotides overlapping the 5′ end of the RBEIII core (underlined in Fig. 1B) matches the Inr consensus (YYYYWCAANKKSY) bound by TFII-I on the rat *XDH/XO* promoter as proposed by Clark et al. [14]. Surprisingly however, an RBEIII-containing oligonucleotide with a 3′ deletion (RBEIII3′Δ) did not efficiently compete for binding of TFII-I to the wild-type RBEIII probe (Fig. 1C, lane 7). In contrast, an RBEIII oligo with a deletion of the 5′ end (RBEIII5′Δ) was able to compete for binding of TFII-I, almost as effectively as the *c-fos* SIE and SRE oligos (Fig. 1C, lane 6). Based on these results, we conclude that TFII-I must primarily interact with residues 3′ of the RBEIII core sequence, rather than with the 5′ flanking sequences that resemble an Inr consensus. To identify specific nucleotides required for binding TFII-I, we performed competitions with a series of mutants of the full RBEIII oligo (Fig. 1D, and not shown). Mutation of the four residues immediately flanking the RBEIII core (mut-G, CATC to gtag, Fig. 1B) completely prevented competition for binding TFII-I (Fig. 1D, lane 4). Of these four residues, substitution of the T residue to A on its own (mut-M, Fig. 1B) noticeably inhibited competition for binding to the wild-type oligo (Fig. 1D, lane 7), which supports the view that TFII-I interacts 3′ of the RBEIII core sequence.

3.2. Nucleotides required for binding of TFII-I are stringently conserved in patients

In our previous analysis, we found that the ACTGCTGA core RBEIII sequence to be as stringently conserved on LTRs amplified from patients [4], and on LTR sequences in Genbank [2], as are the core promoter and enhancer elements. We examined whether nucleotides flanking the RBEIII core are simi-

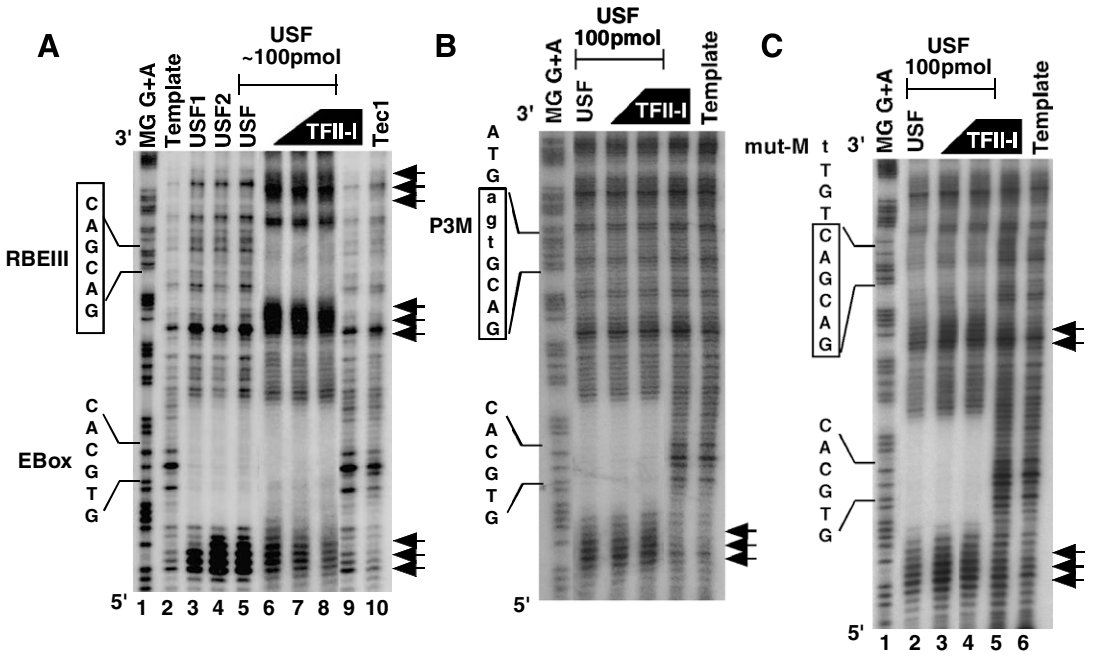

Fig. 2. Specific interaction of TFII-I is required for stimulation of USF binding to RBEIII *Panel A*. DNaseI footprinting reactions were performed with a wild-type LAI HIV-1 LTR fragment, labeled at the 5′ end of the bottom strand. Reactions contained template alone (lane 2), or ~100 pmol USF1 (lane 3), USF2 (lane 4), or USF1/USF2 heterodimers (lanes 5–8) produced by expression in insect cells. TFII-I was added to the reactions at ~25 pmol (lane 6), ~50 pmol (lane 7), or ~100 pmol (lanes 8 and 9). An extract containing the yeast transcription factor TecI was added to the reaction in lane 10. The location of the RBEIII and the E-box motifs were determined by the Maxim and Gilbert G + A chemical cleavage pattern of the naked template (lane 1). DNaseI hypersensitive nucleotides produced by addition of USF are indicated (arrows). *Panels B and C*. Footprinting reactions were performed with the LAI P3M mutant LTR (Panel B) or mut-M LTR fragment (Panel C). Reactions contained template alone (lanes 6), or ~100 pmol USF1/USF2 heterodimers (lanes 2–4). TFII-I was added to the reactions at ~50 pmol (lanes 3), or ~100 pmol (lanes 4 and 5).

larly conserved on LTR sequences from over 200 full-length genomic clone sequences in the NIH HIV sequence database (http://www.hiv.lanl.gov/). Comparable to the previous analysis, the RBEIII core element was conserved in 93% of these sequences, and interestingly, the four nucleotides immediately flanking the core, shown above to be required for binding of TFII-I, were conserved in 99.5% of the isolates (Table 1). Thus, the RBEIII core and immediate 3′ flanking nucleotides are amongst the most stringently conserved *cis*-elements of the viral promoter.

3.3. Specific interaction of TFII-I is necessary for binding of USF to RBEIII in vitro

USF binds with high affinity to Ebox consensus elements (CANNTG), and accordingly addition of recombinant USF on its own to footprinting reactions with the prototypical wild-type LTR causes protection of the upstream Ebox element at −160 (Fig. 2A, lanes 3–5). Consistent with previous experiments using EMSA [2], addition of TFII-I to binding reactions with USF causes additional protection of sequences centered over the RBEIII core element (ACTGCTGA) (lanes 6–8). In contrast, TFII-I added on its own does not cause protection of specific sequences (Fig. 2A, compare lane 9 with lanes 2 and 10). Thus, although specific nucleotides are required for stable interaction of TFII-I with the RBEIII oligo in EMSA, we have not identified conditions where protection of this region can be observed by footprinting. This characteristic of TFII-I has been described previously for binding to the Adenovirus major late (AdML) initiator elements [9]. We also note that binding of USF to the upstream Ebox and RBEIII elements causes the appearance of flanking DNaseI hypersensitivity (indicated with arrows), perhaps indicating formation of a bend. Mutation of the RBEIII core element (P3M), previously shown to prevent binding of RBF2 (USF/TFII-I) in EMSA [2], also prevents interaction of USF with the RBEIII element in footprinting reactions containing both USF and TFII-I (Fig. 2B). Furthermore, the single T–A substitution 3′ flanking the RBEIII core (mut-M), shown above to weaken binding of TFII-I in EMSA, prevents stimulation of USF binding to RBEIII by TFII-I (Fig. 2C, lanes 3 and 4). Reactions with the mut-M template containing both TFII-I and USF do produce some DNAseI hypersensitivity immediately upstream of the RBEIII core, which might be caused by weak association of USF in these reactions (indicated with arrow). These experiments suggest that stable association of TFII-I immediately 3′ of the RBEIII core is required for its ability to stimulate binding of USF to RBEIII in vitro.

3.4. TFII-I bound to the RBEIII is necessary for induction of the integrated LTR

To examine the effect of mutations that prevent binding of TFII-I and USF to the RBEIII site on expression from the LTR in vivo, we used a replication-defective reporter virus (Fig. 3A). VSV-G pseudotyped wild-type and 3′LTR mutant virus was used to infect Jurkat-TAT cells, and integrants were obtained by sorting for expression of an internal eGFP reporter. Approximately equivalent proportions of GFP expressing cells were produced by infections with the wild-type LAI, P3M, and mut-M virus as determined by flow cytometry (Fig. 3B) indicating that the LTR mutants likely do not adversely affect viral integration. Individual clones of eGFP expressing cells were isolated by FACS and expanded for

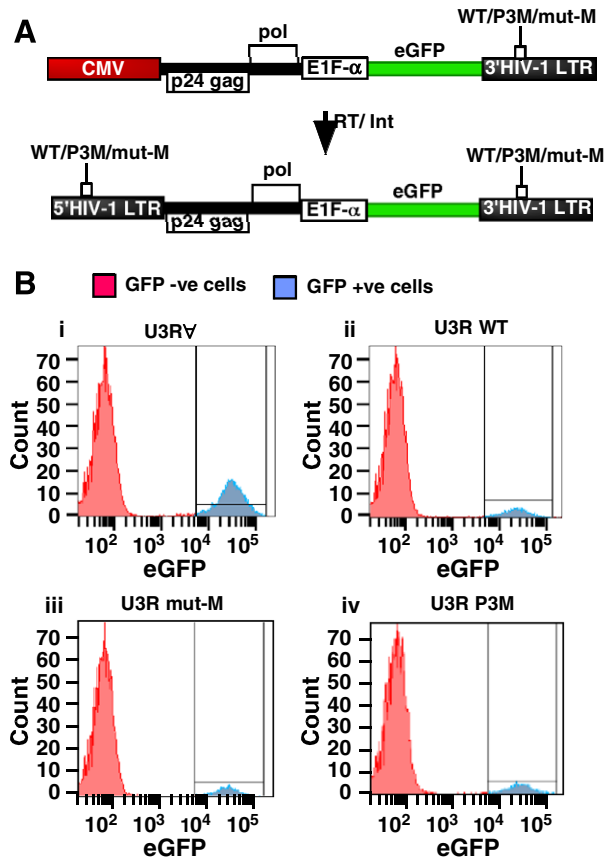

Fig. 3. Reporter virus for assaying effect of LTR mutations on replication. *Panel A*. The self-inactivating 3′ LTR in pTYeGFP was replaced with wild-type, P3M and mut-M LAI LTRs. Following infection with packaged virus, reverse transcription and integration produces integrated reporter virus with 5′ LTR mutations. Integrants can be detected by expression of eGFP from the internal ElF-α promoter. *Panel B*. Jurkat-TAT cells were infected with virus produced from pTYeGFP (i, U3RΔ), pTYeGFP bearing the wild-type (ii, U3R WT), mut-M mutant (iii, U3R mut-M), or P3M mutant LTR (iv, U3R P3M). Flow cytometry to detect eGFP expression was performed 24 h post-infection.

further examination. Clones produced by the wild-type LTR reporter virus typically induced $p24^{gag}$ expression 4–8-fold in response to treatment with PMA/Ionomycin (Fig. 4A). In contrast, clones derived from infection with the P3M or mut-M LTR mutant virus were unresponsive to treatment with PMA/ionomycin (Fig. 4C and D). Additionally, it is evident that both of the mutant LTRs cause an approximately 2-fold higher basal level of $p24^{gag}$ expression relative to the wild-type virus. These observations are consistent with the view that TFII-I and USF bound at RBEIII are essential for induction of latent viral expression in response to T-cell signals. Furthermore, the finding that mutant LTRs generally produce a higher basal signal than wild-type in unstimulated cells is consistent with previous observations using LTR reporter genes integrated by transfection, where we consistently observed elevated basal expression caused by the P3M LTR mutation [2].

3.5. The mut-M mutation prevents interaction of USF and TFII-I with the LTR in vivo

In previous experiments with integrated LTR reporter genes, we have shown that USF1, USF2 and TFII-I are bound to the upstream region of the LTR in unstimulated cells, and remain associated following stimulation with PMA/ionomycin, and also that the P3M RBEIII mutation prevents interaction of these factors [2]. We observe a similar result with the reporter virus, where all three proteins can be detected on the wild-type LTR by ChIP in both unstimulated and stimulated cells (Fig. 5, WT LTR). In contrast, the mut-M mutation, shown above to prevent binding of TFII-I in vitro and induction of LTR expression, also prevents association of USF 1, USF2 and TFII-I with the upstream LTR region in vivo (Fig. 5, mut-M LTR).

4. Discussion

The RBEIII core element and sequences immediately 3′ flanking, shown here to be required for stable association of TFII-I in vitro, are amongst the most highly conserved *cis*-elements on the HIV-1 LTR in patients with AIDS (Table 1). Mutations that prevent binding of TFII-I to this region, including a single nucleotide point substitution, inhibits its ability to stimulate binding of USF to RBEIII in vitro, and also blocks induction of integrated HIV-1 reporter virus by PMA/ionomycin. Additionally, mutations that prevent binding of USF and TFII-I to their upstream sites at RBEIII invariably cause elevated basal expression of integrated provirus [2]. These observations indicate that the combination of TFII-I and USF bound at this upstream location are necessary for induction of transcription in response to T-cell signaling, but also may contribute to repression in unstimulated cells.

The upstream E-box binding site for USF at −160 on the prototypical LAI LTR is not well conserved on sequences from patients with AIDS (Table 1). In this respect, it is interesting that the highly conserved RBEIII site lies only 40 nucleotides further downstream from a non-canonical E-box binding site for USF 1/2. The additional stringent conservation of the immediate 3′ flanking sequences implies that the specific combination of TFII-I and USF bound at this upstream location is critical for the viral replication cycle. USF and TFII-I are likely also bound to RBEI [7], located immediately flanking the core promoter, as though positioned like bookends to the enhancer and core promoter region (Fig. 1). Latent provirus is known to have nucleosomes positioned immediately upstream of −160 and downstream of the initiator, and it is possible that USF/TFII-I contribute to positioning and modification of these histones. Alternatively, or additionally, considering the requirement of MAP kinase signaling for induction of the integrated LTR [3], binding of TFII-I to its specific upstream site might be required to "deliver" activated MAP kinases for regulation of factors bound to the adjacent enhancer [17]. TFII-I interacts with multiple different sequence specific DNA binding factors, including in addition to USF, Myc, Phoxl, SRF, STAT1 and STAT3 (reviewed in [1]). The results presented here demonstrate that the USF–TFII-I interaction produces a unique specificity, and consequently it is possible that TFII-I may also promote binding of these additional factors to non-canonical *cis*-elements. This may represent an additional largely unrecognized function for TFII-I.

Acknowledgements: This research was supported by funds from the Canadian Institutes for Health Research (MOP-77807). We thank Brendan Bell for useful discussions, and Andy Johnson for expert assistance with FACS analysis.

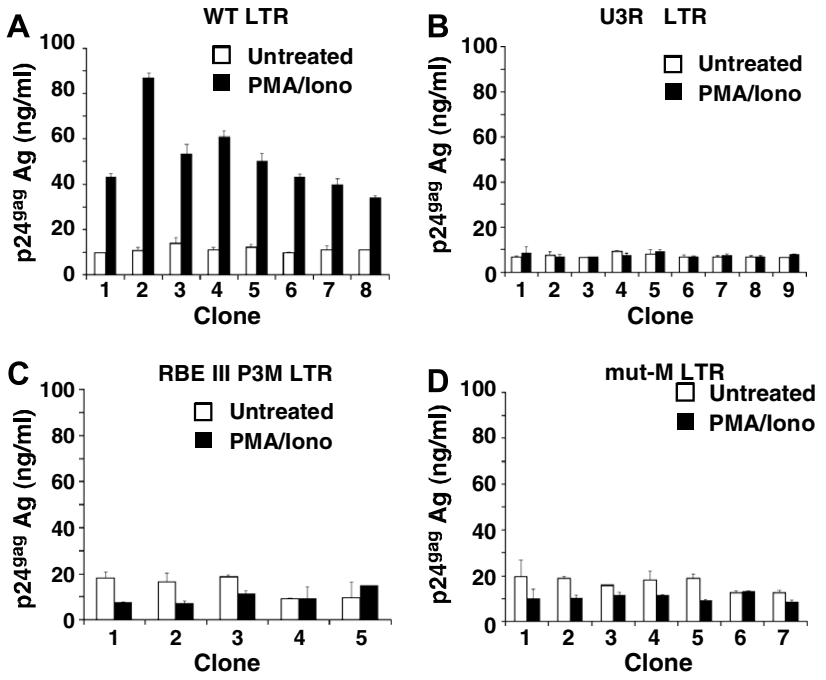

Fig. 4. Mutations that prevent binding of TFII-I and USF1/2 at RBEIII prevent induction of LTR transcription. Jurkat-TAT cells were infected with wild-type LTR reporter virus (*Panel A*), pTYeGFP U3RΔ virus (*Panel B*), P3M mutant LTR (*Panel C*), or mut-M LTR mutant reporter virus (*Panel D*), and eGFP expressing clones isolated by FACS. Cells were unstimulated (open bars) or stimulated with PMA/ionomycin (closed bars) for 24 h, prior to assaying $p24^{gag}$ expression by ELISA. Results represent the average of assays from three independent cultures.

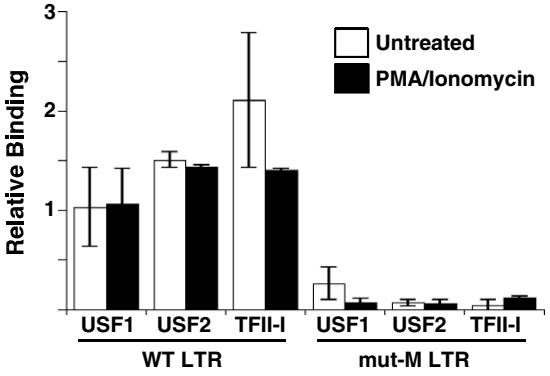

Fig. 5. The mut-M mutation prevents interaction of USF1, USF2 and TFII-I with the LTR in vivo. Chromatin immunopreciptiation was performed with USF1, USF2 and TFII-I antibodies on Jurkat-TAT clones bearing a wild-type LTR integrated reporter virus (clone 3, WT LTR), or the mut-M LTR mutant (clone 4, mutM LTR). Prior to cross-linking with formaldehyde, the cells were left untreated (open bars), or stimulated with PMA and ionomycin for 4 h (closed bars). Semi-quantitative PCR was performed with LTR- and β-globin-specific primers, and the fold enrichment of immunoprecipitated LTR template relative to input was normalized to the β-globin controls. The results represent an average of three determinations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.10.032.

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