OTK18, a zinc-finger protein, regulates human immunodeficiency virus type 1 long terminal repeat through two distinct regulatory regions

Masahide Horiba,1,2 Lindsey B. Martinez,1,2 James L. Buescher,1,2 Shinji Sato,1,2 Jenae Limoges,3 Yunquan Jiang,4 Clinton Jones4 and Tsuneya Ikezu1,2

Correspondence
Tsuneya Ikezu
tikezu@unmc.edu

1Center for Neurovirology and Neurodegenerative Disorders, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA
2Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA
3Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5880, USA
4Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, NE 68583-0905, USA

Received 24 March 2006
Accepted 29 August 2006

It has previously been shown by our laboratory that OTK18, a human immunodeficiency virus (HIV)-inducible zinc-finger protein, reduces progeny-virion production in infected human macrophages. OTK18 antiviral activity is mediated through suppression of Tat-induced HIV-1 long terminal repeat (LTR) promoter activity. Through the use of LTR-scanning mutant vectors, the specific regions responsible for OTK18-mediated LTR suppression have been defined. Two different LTR regions were identified as potential OTK18-binding sites by an enhanced DNA–transcription factor ELISA system; the negative-regulatory element (NRE) at \(-255/-238\) and the Ets-binding site (EBS) at \(-150/-139\) in the LTR. In addition, deletion of the EBS in the LTR blocked OTK18-mediated LTR suppression. These data indicate that OTK18 suppresses LTR activity through two distinct regulatory elements. Spontaneous mutations in these regions might enable HIV-1 to escape from OTK18 antiretroviral activity in human macrophages.
of brain mononuclear phagocytes in severe HIV-1 encepha-
ritis and may serve as a ‘surrogate’ marker for HIV-1-
associated dementia (Carlson et al., 2004b). Elucidation of
OTK18-binding sequences within the HIV-1 LTR is critical
for the molecular characterization of OTK18 antiviral
activity.

In this study, we employed all 27 LTR promoter linker-
screening mutants fused to the luciferase gene to screen for
potential OTK18 response elements in the LTR (−453/
+18) of the HXB2 clone (Zeichner et al., 1991). The linkerscanning mutants consecutively replaced 18 bp of wild-
type sequence with an NdeI–Xhol–SalI (NNS) poly linker
(CATATGTCGAGGTCGAC) across the U3 and R regions.
Human embryonic kidney 293 cells (10⁵ cells per well on 24-
well plates; Fisher Scientific) were co-transfected with the
LTR-scanning mutant luciferase vectors (300 ng), Tat1–72
expression vector (pSV2Tat72, 50 ng) (Subramani
et al., 1981), a Renilla luciferase reference construct (pTK-RL,
50 ng) and the OTK18 expression vector (pcDNA-OTK18,
1 μg) by using GenePorter (Gene Therapy Systems) as
described previously (Carlson et al., 2004a). Forty-eight
hours after transfection, cells were collected and luciferase
activity was measured by using a luminometer (Berthold
systems) and we chose 2 μg nuclear extract for the follow-
ing experiment.

The OTK18-binding activity to each element and its
sensitivity to cold probe ranging from 1 × 10⁰ to 100 × was
tested (Fig. 1c). The oligonucleotide pairs used for double-
stranded DNA probes are A3 [biotin-(N×100)-CCTG-
GATCTGTGGATCTA and TAGATCCACAGATCAAGG],
A8 [biotin-(N×100)-TGATGTGGCTACAAGTCT and
AGCTTGATGCACCATTCA], A10 [biotin-(N×100)-GAAGT-
TAGAGAAGGCAAA and TTGGCTTCTTCTACATCT], A12
[bio tin-(N×100)-CTTGTACACTCATTTCG and AGCT-
CACAGGTTGTAACAG], B1 [biotin-(N×100)-CTTGT-
GAAGTTAGCAAG and TCTAAGTTACTCAT], B6
[biotin-(N×100)-CTCGAGCTACAGTCAAGA and TTCC-
TTAGTTACTCCGG] and EBS [biotin-(N×100)-CATC-
CGGAG and CTCCGGATG]. OTK18 binding was specific,
as significant binding occurred only in the presence of
OTK18-infected cell lysate and not in the presence of lysis
buffer alone or uninfected cell lysate (Fig. 1d). Whilst
regions A3, A8 and B1 demonstrated significant binding to
OTK18, none of them was outcompeted significantly by
cold probes in a dose-dependent manner (Fig. 1d). Only the
A12 and EBS elements showed significant binding to
OTK18, which was outcompeted by cold probes. The B1
element showed OTK18 binding, but was not outcompeted
by cold probe, and B6, which contains partial EBS sequence,
had no binding activity. In addition, none of the binding was
outcompeted by up to 100-fold excess of single-stranded
oligonucleotides corresponding to the binding sequence,
suggesting its specificity to double-stranded DNA (data not
shown). These data indicate that the A12 and EBS elements
are potential specific OTK18-binding sites on the HIV-1
LTR.

The EBS is known to be an important response element for
the cooperative interaction of Ets-1 with the upstream
stimulatory factor (USF)-1 in HIV-1 enhancer activity
(Sieweke et al., 1998). As the B6 region does not cover EBS
completely, we created an HXB2-derived LTR luciferase
vector lacking EBS (−150/−139) (pLTR-EBS-Luc) with a
modified QuikChange II site-directed mutagenesis kit.
Table 1. Suppression of LTR linker-scanning mutants by OTK18

<table>
<thead>
<tr>
<th>Region</th>
<th>Location</th>
<th>OTK18 suppression*</th>
<th>Transcriptional element</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>−453/−436</td>
<td>+ +</td>
<td>Site A</td>
</tr>
<tr>
<td>A2</td>
<td>−435/−418</td>
<td>+ +</td>
<td>AP-1</td>
</tr>
<tr>
<td>A3</td>
<td>−417/−400</td>
<td>−</td>
<td>NRE</td>
</tr>
<tr>
<td>A4</td>
<td>−399/−382</td>
<td>+ +</td>
<td>NRE</td>
</tr>
<tr>
<td>A5</td>
<td>−381/−364</td>
<td>+</td>
<td>NRE, NFAT-1</td>
</tr>
<tr>
<td>A6</td>
<td>−363/−346</td>
<td>+</td>
<td>NRE, NFAT-1, IL-2</td>
</tr>
<tr>
<td>A7</td>
<td>−345/−328</td>
<td>+ +</td>
<td>NRE, IL-2</td>
</tr>
<tr>
<td>A8</td>
<td>−327/−310</td>
<td>−</td>
<td>NRE</td>
</tr>
<tr>
<td>A9</td>
<td>−309/−292</td>
<td>+</td>
<td>NRE</td>
</tr>
<tr>
<td>A10</td>
<td>−291/−274</td>
<td>−</td>
<td>NRE, NFAT-1</td>
</tr>
<tr>
<td>A11</td>
<td>−273/−256</td>
<td>+ +</td>
<td>NRE, NFAT-1, IL-2</td>
</tr>
<tr>
<td>A12</td>
<td>−255/−238</td>
<td>−</td>
<td>NRE, IL-2</td>
</tr>
<tr>
<td>B1</td>
<td>−237/−220</td>
<td>−</td>
<td>NRE, IL-2</td>
</tr>
<tr>
<td>B2</td>
<td>−219/−202</td>
<td>+ +</td>
<td>NRE</td>
</tr>
<tr>
<td>B3</td>
<td>−201/−184</td>
<td>+ +</td>
<td>NRE</td>
</tr>
<tr>
<td>B4</td>
<td>−183/−166</td>
<td>+ +</td>
<td>C/EBP II</td>
</tr>
<tr>
<td>B5</td>
<td>−165/−148</td>
<td>+ +</td>
<td>USF-1</td>
</tr>
<tr>
<td>B6</td>
<td>−147/−130</td>
<td>−</td>
<td>Ets, LEF-1</td>
</tr>
<tr>
<td>B7</td>
<td>−129/−112</td>
<td>+ +</td>
<td>LEF-1, C/EBP II</td>
</tr>
<tr>
<td>B8</td>
<td>−111/−94</td>
<td>+ +</td>
<td>NF-κB, HIVEN86A, EBP-1</td>
</tr>
<tr>
<td>B9</td>
<td>−93/−76</td>
<td>NA</td>
<td>NF-κB, HIVEN86A, EBP-1</td>
</tr>
<tr>
<td>B10</td>
<td>−75/−58</td>
<td>NA</td>
<td>Sp1</td>
</tr>
<tr>
<td>B11</td>
<td>−57/−40</td>
<td>NA</td>
<td>Sp1</td>
</tr>
<tr>
<td>B12</td>
<td>−39/−22</td>
<td>NA</td>
<td>TATA</td>
</tr>
<tr>
<td>C1</td>
<td>−21/−4</td>
<td>NA</td>
<td>LBP-1, UBP-1</td>
</tr>
<tr>
<td>C2</td>
<td>−3/+15</td>
<td>+</td>
<td>LBP-1, UBP-1</td>
</tr>
<tr>
<td>C4</td>
<td>−105/−81</td>
<td>+</td>
<td>NF-κB</td>
</tr>
</tbody>
</table>

*Suppression of pSVTat72-mediated LTR-luciferase expression by OTK18; −, + and + + denote no suppression, 50–79 % suppression and 80–99 % suppression, respectively. NA, No detectable LTR activation by Tat and no suppression by OTK18.

(Stratagene) using oligonucleotide pairs and tested its promoter activity in the presence/absence of HIV-1 Tat and OTK18 in 293 cells (Fig. 2). Unexpectedly, pLTRAEBS-Luc showed enhanced luciferase activity in the presence of Tat and OTK18 (second column) compared with Tat alone (first column). Tat-activated luciferase activity of the original LTR-Luc, on the other hand, was suppressed significantly by OTK18 (Fig. 2b). This suggested that EBS is a critical element for the OTK18-mediated LTR suppression and that OTK18 has a dual regulatory function in the HIV-1 LTR, dependent on specific binding regions (see below). As the proximal promoter region between nucleosomes nuc0 and nuc1, where EBS is located, has been established as a critical regulatory region, EBS is an important site for the suppressive effect of OTK18. All of the above data indicate that EBS (−150/−139) is the primary OTK18 response element on the HIV-1 LTR.

We have shown previously that the transcriptional factor OTK18 suppresses both HIV-1 Tat-mediated LTR activation in vitro and HIV-1 replication in human monocyte-derived macrophages (MDMs) (Carlson et al., 2004a). However, the response element for OTK18 suppression has not been determined. We have shown that OTK18 interacts with two distinct regions on the HIV-1 LTR, the NRE and EBS regions, by using LTR-scanning mutants and EMSA and through more defined subcloning/deletional analyses. Multiple regions of the NRE have been reported to downregulate HIV transcription (Garcia et al., 1987). Specifically, NRE (−182/−153) contains a binding site for the nuclear factors USF (Sawadogo et al., 1988), NFIL-6 (Tesmer et al., 1993) and human GATA-3 (Yang & Engel, 1993; Galio et al., 1999). Furthermore, NRE (−220/−160) binds to nuclear matrix proteins and inhibits NF-κB activity (Hoover et al., 1996). However, no proteins have been identified that bind to NRE (−255/−238). Thus, OTK18, to the best of our knowledge, is the first cellular protein to be shown to interact with this specific region of the NRE and suppress the HIV-1 LTR.

A number of mutations were reported at this region in LTRs derived from human genomic DNA of HIV-1-infected patients. Estable et al. (1996) reported LTR-proximal sequences from 42 HIV-1-infected cases ranging from
stage I to IV patients (World Health Organization staging I–IV). Although they concluded that the Ets core sequence (ATCCG) was highly conserved, 25 of a total of 60 LTR sequences from the 42 cases were mutated in the Ets-1 element. The Ets core sequence was highly conserved in non-B subtypes (De Arellano et al., 2005). We have also examined the available LTR depository at the Los Alamos HIV sequence database for LTRs of A, B, C and D subtypes. The conservation of ETS core sequence (ATCCG) was 19/19 (100 %, A), 45/48 (94 %, B), 73/74 (97 %, C) and (90 %, D).

---

**Fig. 1.** DNA ELISA screening for binding of OTK18 to HIV-1 LTR regions. (a, b) Improved chemiluminescent method for DNA–transcription factor ELISA. Sensitivity of two methods, conventional colorimetry (a) and chemiluminescence (b), was compared by using varying amounts of OTK18 protein extracts bound to a negative-control oligonucleotide (empty bars) or a double-stranded oligonucleotide corresponding to the A8 region of the HIV-1 LTR region (filled bars). Statistical analysis was performed by using one-way ANOVA to compare binding of each amount of extract with the binding of the negative-control oligonucleotide for the corresponding amount of extract (*P < 0.001; §P < 0.05). (c) Six regions of the HIV-1 LTR (A3–B6 and EBS) were screened for specific binding of the OTK18 protein. Binding was compared in the presence of lysis buffer alone (no lysate, filled bars), uninfected Sf9 cell lysate (uninfected lysate, shaded bars) or OTK18 baculovirus-infected lysate (OTK18 lysate, empty bars). Only the OTK18 lysate showed significant binding (*P < 0.001 compared with no lysate or uninfected lysate). (d) Binding was tested in the presence of no extract (–, empty bars), extract alone with no competing oligonucleotide (0 x, filled bars), 1 x excess of the corresponding LTR oligonucleotide (checked bars), 5 x excess oligonucleotide (diagonally hatched bars), 20 x excess oligonucleotide (horizontally hatched bars) or 100 x oligonucleotide (shaded bars). Statistical analysis was performed by using one-way ANOVA to compare binding of each oligonucleotide with no competitor present to the corresponding no-extract condition (#P < 0.001). Statistical analysis was also performed by using one-way ANOVA to compare binding of the competition conditions with the no-competitor condition for the same oligonucleotide (†P < 0.01; #P < 0.001). NC, Negative control; RLU, relative luciferase units.
subtypes impact their suppression by OTK18 and if such an impact is required to understand whether LTRs derived from different homology domains in these different OTK18 isoforms. Further study is necessary in order to characterize the expression of these OTK18 isoforms in MDMs and their respective roles in HIV-1 replication and LTR regulation.

Acknowledgements

We would like to thank Drs A. Ghorpade and J. West for critical reading of the manuscript, the NIH AIDS Research and Reference Reagent Program for pSV2Tat72 (Alan Flankel) and LTR linker-scanning mutants (Steve Zeichner), S. Wakeley and G. Weber for technical assistance and R. Taylor for editorial support. This work is supported by NIH grants R01 AI5089401, R01 MH072539 (T. L.), K08 MH01552 (J. L.) and NCRR P20RR15635 (T. I. and C. J.).

References


The common Ets sequence was TGCACTCCGGAG (89 % in A, 73 % in B, 3 % in C and 38 % in D), followed by TACATCCGGGAG (5 % in A, 4 % in B, 77 % in C and 13 % in D). The most striking difference is the specific dominance of TACATCCGGGAG in subtype C, which will be worthwile to pursue for future study (the mutation TAC in type C is at position −149, whereas the beginning of the core Ets sequence is at −147). Further investigation is required to understand whether LTRs derived from different subtypes impact their suppression by OTK18 and if such an impact is attributed to the difference in the Ets sequence.

The upregulation of LTRΔEBS by OTK18 in the presence, but not in the absence, of Tat is unexpected, but it indicates that OTK18 may have a dual role in LTR regulation. We have found previously that there are two forms of OTK18 (Carlson et al., 2004a), which we define as 75 kDa OTK18α and 65 kDa OTK18β. OTK18β lacks the KRAB-A box and may not act as a transcriptional suppressor. Thus, we believe that the dual gene regulation conferred by OTK18α and OTK18β may arise due to the presence or absence of different homology domains in these different OTK18 isoforms.


