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

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Summary

We have previously shown 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 viral long terminal repeat (LTR) promoter activity. Through the use of LTR scanning mutant vectors, we have defined the specific regions responsible for OTK18-mediated LTR suppression. 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 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.

The control of *human immunodeficiency virus* (HIV) type 1 entry, reverse transcription, integration, expression, and production are operated by distinct molecular mechanisms, which have been targeted by antiretroviral therapy. Among these mechanisms, nuclear factors, such as NFκB and 45/39kD CCAAT/enhancer binding protein-β (C/EBPβ) isoforms, are important in regulating HIV-1 expression (Griffin *et al.*, 1989; Henderson *et al.*, 1996). In addition, several transcription factors can repress viral transcription, often by binding to specific sequences contained in the viral long terminal repeat (LTR) (Patarca *et al.*, 1988; Subler *et al.*, 1994; Ray & Srinivas, 1997; Weiden *et al.*, 2000; Cicala *et al.*, 2002; Hayes *et al.*, 2002). Recently, we have shown that the transcriptional factor OTK18 is induced by and suppresses HIV-1 infection in mononuclear cells (K. Carlson *et al.*, 2004). OTK18 is classified as a transcription factor as it contains 13 C_2H_2-type DNA binding zinc finger motifs (Saito et al., 1996). C_2H_2 zinc finger motifs are capable of binding to a wide range of DNA sequences, including the HIV-1 LTR (Wu *et al.*, 1995; Isalan *et al.*, 2001).

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We have previously demonstrated that one putative anti-viral mechanism of OTK18 involves direct suppression of the LTR (K. Carlson *et al.*, 2004). Mutational analyses revealed that the suppressive activity of OTK18 lies within amino acids 26-89. This region shares homology with a family of zinc-finger proteins containing the Krüppel-associated box (KRAB) motif, a repression domain encoded by numerous transcription factors. Pengue *et al* found that the KRAB domain fused to HIV-1 Tat was able to repress basal HIV-1 promoter activity in HeLa cells (Pengue et al., 1995). This finding was further supported by the demonstration that a KRAB-containing tetracycline-binding protein can suppress HIV-1 replication through randomly integrated tetracycline response elements within the HIV-1 genomic sequence (Herchenroder et al., 1999). In addition, Reynolds *et al*. reported that a genetically engineered KRAB domain containing a C_2H_2 type zinc finger protein could suppress Tat-mediated HIV-1 LTR activity, thereby making it an attractive candidate for antiretroviral therapy (Reynolds et al., 2003). In this context, OTK18 is the first KRAB-containing C_2H_2 zinc finger protein endogenously expressed in macrophages that has antiviral activity. Interestingly, OTK18 is specifically expressed in the cytosol of brain MP in severe HIV-1 encephalitis and may serve as a "surrogate" marker for HAD (K. A. Carlson *et al.*, 2004). 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-scanning 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 linker-scanning mutants consecutively replaced 18 bp of wild-type sequence with an NdeI-XhoI-SalI(NXS) polylinker (CATATGCTCGAGGTCGAC) across the U3 and R regions. Human embryonic kidney 293 cells (10^5 cells/well on 24-well plates, Fisher Scientific) were co-transfected with the LTR scanning mutant luciferase vectors (300 ng), Tat1-72 expression vector (pSV2tat72, 50ng) (Subramani et al., 1981), a *Renilla* luciferase reference construct (pTK-RL, 50ng) and the OTK18 expression vector (pcDNA-OTK18, 1μg) using GenePorter (Gene Therapy Systems) as previously described (K. Carlson *et al.*, 2004). Forty-eight hours after transfection, cells were collected and luciferase activity was measured by luminometer (Berthold Systems Inc., Aliquippa, PA) using the Dual-Luciferase kit (Promega). As shown in Table 1, we identified six regions (A3, A8, A10, A12, B1, and B6, corresponding -417/-400, -327/−310, -291/−274, -255/−238, -237/−220, and -147/−130), which were resistant to the OTK18-mediated gene suppression in the presence of Tat. These codes correspond to the original codes by Zeichner et al.

The binding of OTK18 to each of these six regions was tested by a novel DNA-trasnscriptional factor ELISA, which is about 10-fold more sensitive than conventional electromobility gel shift analysis (EMSA). Development of such a technique was necessary since binding of OTK18 to double strand oligonucleotides corresponding to the regions were too weak to be examined by conventional EMSA (data not shown). For that purpose, we generated a baculovirus expressing 6×His-Express-tagged full-length OTK18 by in-frame insertion of full-length OTK18 gene into pBlueBacHis2A vector (Invitrogen) at the Bam HI site. The resultant vector (pBlueBacHis2A-OTK18) was inserted into Bac-N-Blue AcMNPV DNA by homologous recombination according to the manufacturer's procedure. Infection of sf9 insect cells with the optimized titer of OTK18 baculovirus resulted in expression of recombinant OTK18 at 84 hr post-infection and detected as 75kD protein (data not shown). OTK18 protein was specifically collected from the nuclear extract fraction, which we used for subsequent experiments as control nuclear extract.

DNA-transcriptional factor ELISA method was originally described by Reynolds *et al* (Reynolds et al., 2003) and is commercially available as the colorimetric ELISA TransAM kit (Active Motif). To enhance the sensitivity of the original protocol, we incorporated

luminol-based conversion of hydrogen peroxidase activity to chemiluminescence using SuperSignal ELISA Pico Chemiluminescent Substrate (Pierce) instead of conventional chromogenic development. As shown in Figure 1, the colorimetric ELISA shows statistically significant difference between a nonspecific oligo (control) and the HIV-1 LTR oligo A8 in the presence of OTK18-containing nuclear extract from 0.5 to 5 μg input (Fig. 1A), but the signal-to-noise (S/N) ratio is 1.31-1.41 and Z′ factors are −5.64 – 0.045. In case of our luminescence system, there is statistical significance between two groups from 0.5 to 5 μg input (Fig. 1B) with S/N ratio from 1.83 – 2.59 and Z′ factors −0.88 to 0.85, which was calculated as per Zhang and others (Zhang et al., 1999). Since higher S/N ratio and Z′ factor indicate suitability of assay system, we conclude that our luminescence system is superior to colorimetric system, and we chose 2 μg nuclear extract for the following experiment.

The OTK18 binding activity to each element and its sensitivity to cold probe ranging from 1× to 100× was tested (Fig. 1C). The oligonucleotide pairs used for double stranded DNA probes are A3 (Biotin-(Nx100)-CCTTGATCTGTGGATCTA and TAGATCCACAGATCAAGG), A8 (Biotin-(Nx100)-TGGATGGTGCTACAAGCT and AGCTTGTAGCACCATCCA), A10 (Biotin-(Nx100)-GAAGTTAGAAGAAGCCAA and TTGGCTTCTTCTAACTTC), A12 (Biotin-(Nx100)-CTTGTTACACCCTGTGAG and CTCACAGGGTGTAACAAG), B1 (Biotin-(Nx100)-CCTGCATGGAATGGATGA and TCATCCATTCCATGCAGG), B6 (Biotin-(Nx100)-TCCGGAGTACTTCAAGAA and TTCTTGAAGTACTCCGG), and EBS (Biotin-(Nx100)-CATCCGGAG 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). While A3, A8, and B1 region demonstrated significant binding to OTK18, none of them was significantly competed by cold probes in dose-dependent manner (Fig. 1D). Only A12 and EBS elements showed significant binding to OTK18, which were competed by cold probes. B1 element showed OTK18 binding, but was not competed by cold probe, and B6, which contains partial EBS sequence, had no binding activity. In addition, none of the binding was competed by up to 100× fold excess amount of single-strand oligonucleotides corresponding to the binding sequence, suggesting its specificity to dsDNA (data not shown). These data indicate that A12 and EBS elements are the potential specific OTK18 binding sites on 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). Since the B6 region does not completely cover EBS, we created a HXB2-derived LTR luciferase vector lacking EBS (−150/−139) (pLTRΔEBS-Luc) with a modified QuickChange II site-directed mutagenesis kit (Stratagene) using oligonucleotide pairs and tested its promoter activity in the presence/absence of HIV-1 Tat and OTK18 in 293 cells (Figure 2). Unexpectedly, (pLTRΔEBS-Luc) showed enhanced luciferase activity in the presence of Tat and OTK18 (second column) as compared to Tat alone (first column). Tat-activated luciferase activity of original LTR-Luc, on the other hand, was significantly suppressed 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). Since the proximal promoter region between nucleosomes nuc0 and nuc1, where EBS is located has been established as a critical regulatory region, EBS is important site for the suppressive effect of OTK18. All the above data indicate that EBS (−150/−139) is the primary OTK18 response elements on the HIV-1 LTR.

We have previously shown that the transcriptional factor OTK18 suppresses both HIV-1Tat-mediated LTR activation *in vitro* and HIV-1 replication in human MDM (K. Carlson *et al.*, 2004). However, the response element for OTK18 suppression has not been determined. We

have shown that OTK18 interacts with two distinct regions on HIV-1 LTR, the NRE and EBS regions, using LTR scanning mutants, EMSA, and through a more defined subcloning/deletional analyses. Multiple regions of the NRE have been reported to down-regulate 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 NFκB 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.* (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 from I to 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 are 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). The common Ets sequence was TGCATCCGGAG (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 worthwhile to pursue for the future study (the mutation TAC in type C is at position −149 where the beginning of the core ets sequence is at −147). Further investigation is required to understand if 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 up-regulation 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 previously found that there are two forms of OTK18 (K. Carlson *et al.*, 2004), which we define as 75kD OTK18α and 65kD 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. Further study is necessary in order to characterize the expression of these OTK18 isoforms in MDM and their respective roles in HIV-1 replication and LTR regulation.

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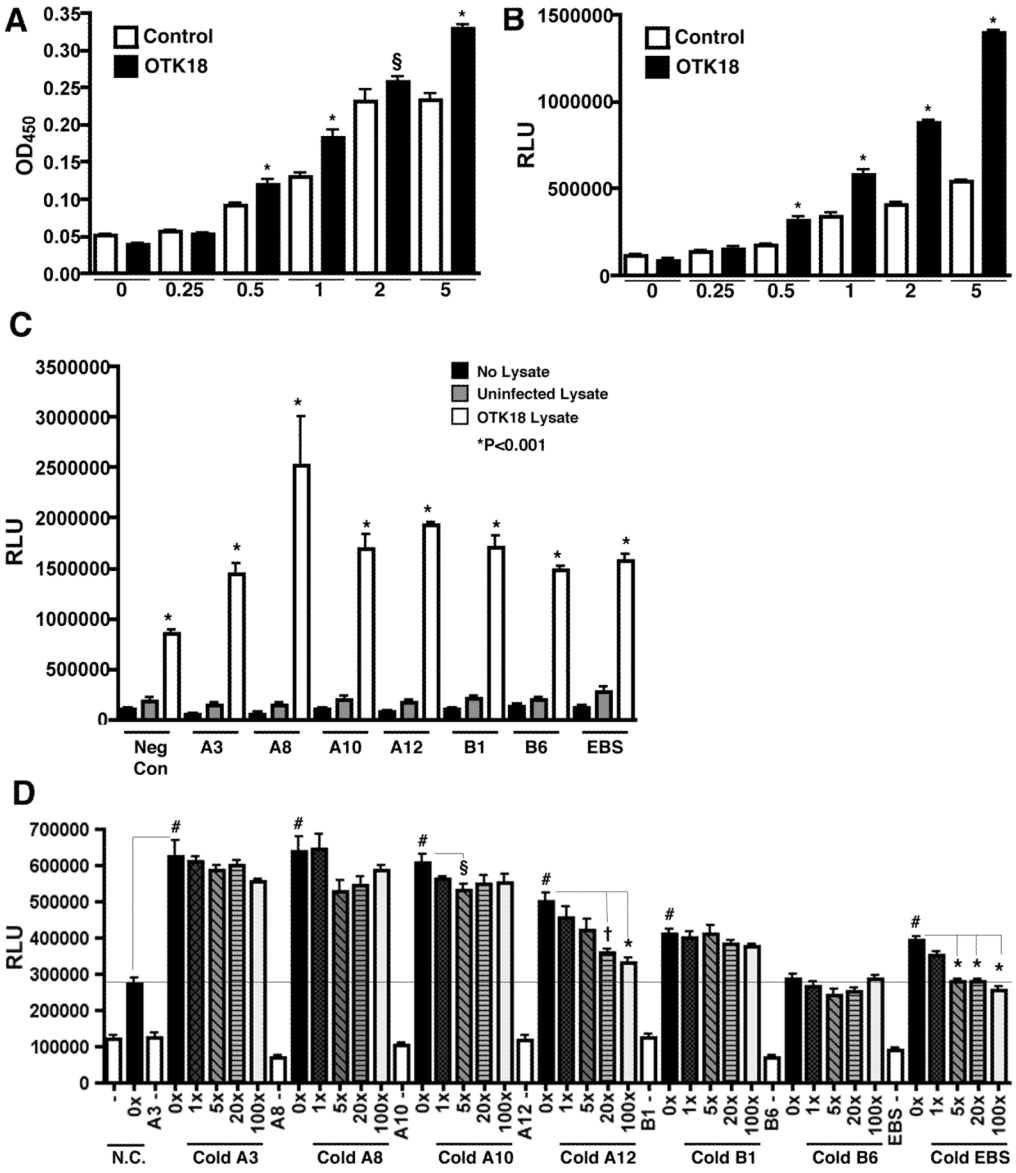

Figure 1. DNA ELISA screening for binding of OTK18 to HIV-1 LTR regions

(A-B) Improved Chemiluminescent Method for DNA-Transcriptional Factor ELISA. Sensitivity of two methods, conventional colorimetry (A) and chemiluminescence (B), were compared using varying amounts of OTK18 protein extracts bound to a negative control oligo (white column) or a double-stranded oligo corresponding to the A8 region of the HIV-1 LTR region (black column). Statistical analysis was performed using one-way ANOVA to compare binding of each amount of extract to the binding of the negative control oligo for the corresponding amount of extract (* indicates $p<0.001$; § indicates $p<0.05$). (C) Six regions of the HIV-1 LTR (A3-B6 and EBS) were screened for specific binding of OTK18 protein. Binding was compared in the presence of lysis buffer alone (No Lysate, black column), uninfected SF9 cell lysate (Uninfected Lysate, gray column), or OTK18 baculovirus-infected lysate (OTK18 Lysate, white column). Only the OTK18 lysate showed significant binding (* indicates $p<0.001$ as compared to no lysate or uninfected lysate). (D) Binding was tested in the presence of no extract (-, white column), extract alone with no competing oligo (0×, black column), 1× excess of the corresponding LTR oligo (checked

column), 5× excess oligo (diagonal stripes), 20× excess oligo (horizontal stripes), or 100× oligo (gray column). Statistical analysis was performed using one-way ANOVA to compare binding of each oligo with no competitor present to the corresponding no extract condition (# indicates $p<0.001$). Statistical analysis was also performed using one-way ANOVA to compare binding of the competition conditions to the no competitor condition for the same oligo († indicates $p<0.01$; # indicates $p<0.001$).

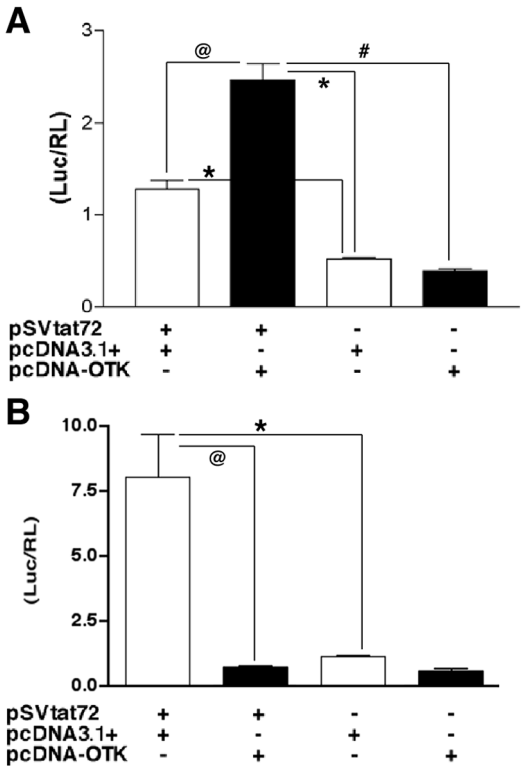

Figure 2. Luciferase assay of LTR elements and LTRΔEBS

A. HEK293 cells were transfected with pSVtat72, pLTRΔEBS-Luc (firefly luciferase), pTK-RL (*Renilla* luciferase) and either pcDNA3.1 or pcDNA-OTK18. Transcriptional activity was expressed as a ratio of the reporter gene (pLTRΔEBS-Luc) to the reference gene (pTK-RL). B. HXB2 derived original LTR-Luc was tested in the same experimental design. *, #, and @ denote $p<0.05$ vs. Tat(-) pcDNA3.1, Tat(-) pcDNA-OTK18, or Tat(+) pcDNA3.1, respectively.

Table 1
Suppression of LTR linker-scanning mutants by OTK18

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

Note.

[a] Suppression of pSVTat72 mediated LTR-luciferase expression by OTK18; -, +, and ++ denote no suppression, 50-79% suppression, and 80-99% suppression, respectively. NA denotes no detectable LTR activation by Tat and no suppression by OTK18.