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# Functional Studies of CCAAT/Enhancer Binding Protein Site Located Downstream of the Transcriptional Start Site

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**ABSTRACT:** Previous studies have identified a CCAAT/enhancer binding protein (C/EBP) site located downstream of the transcriptional start site (DS3). The role of the DS3 element with respect to HIV-1 transactivation by Tat and viral replication has not been characterized. We have demonstrated that DS3 was a functional C/EBP $\beta$  binding site and mutation of this site to the C/EBP knockout DS3-9C variant showed lower HIV-1 long terminal repeat (LTR) transactivation by C/EBP $\beta$ . However, it was able to exhibit similar or even higher transcription levels by Tat compared to the parental LTR. C/EBP $\beta$  and Tat together further enhanced the transcription level of the parental LAI-LTR and DS3-9C LTR, with higher levels in the DS3-9C LTR. HIV molecular clone viruses carrying the DS3-9C variant LTR demonstrated a decreased replication capacity and delayed rate of replication. These results suggest that DS3 plays a role in virus transcriptional initiation and provides new insight into C/EBP regulation of HIV-1.

**KEYWORDS:** HIV-1, C/EBP, Tat, transcription

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## Introduction

Human immunodeficiency virus type 1 (HIV-1) gene expression in cells of the monocyte–macrophage lineage has been shown to be critically dependent on the regulation of the long terminal repeat (LTR), the promoter that drives proviral gene expression from the integrated viral DNA template. In turn, LTR activation requires cellular transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B), CCAAT/enhancer binding protein (C/EBP), Sp1, and activating transcription factor/cyclic AMP response element binding protein (ATF/CREB), and a number of other transcription factors as previously reviewed.<sup>1–3</sup> Four C/EBP binding sites have been identified within the HIV-1 subtype B LTR, three located upstream of the HIV-1 LTR transcriptional start site (C/EBP US1, US2, US3),<sup>4</sup> and one located downstream of the transcriptional start site (C/EBP-DS3).<sup>5</sup> A number of studies have characterized the functional properties of the two upstream C/EBP binding sites, C/EBP US1 and US2, which are required for HIV-1 replication in cells of the monocyte–macrophage lineage but not for replication in T-cells.<sup>6,7</sup> Additionally, specific sequence configurations of C/EBP US1 have been shown to correlate with the development of HIV-1-associated dementia (HAD) and

disease progression.<sup>8–10</sup> The C/EBP family has been shown to be composed of at least six different proteins (C/EBP- $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ ) and belongs to the basic leucine zipper transcription factor family.<sup>11</sup> C/EBP $\beta$  has been reported to regulate HIV-1 transcription in different cell types in association with a number of cellular factors including Sp1, NF- $\kappa$ B, ATF/CREB, and CBP/p300 and the viral proteins, such as Tat and Vpr.<sup>8,12–21</sup>

The HIV-1 Tat protein is an 81–101 amino acid protein that has been shown to be necessary for HIV-1 replication and transcription as previously reviewed.<sup>22–25</sup> Tat has been shown to mediate transactivation of HIV-1 through binding to the Tat-activated region at the 5' end of all HIV-1 mRNAs. The interaction of Tat with Tat-activated region results in the recruitment of positive transcriptional elongation factor (p-TEF), composed of cyclin-dependent kinase 9 (cdk9), and its partner, cyclin T1.<sup>26,27</sup> p-TEF is responsible for phosphorylation of the C-terminal domain of RNA polymerase II (RNA Pol II) and promotes transcription elongation.<sup>28,29</sup> Tat was able to regulate the activity of HIV-1 LTR by interacting with a number of proteins, including Sp1, NF- $\kappa$ B, C/EBP $\beta$ , and CBP/p300.<sup>30–35</sup> Specifically, Tat directly binds to C/EBP $\beta$



in vitro and in vivo through amino acid residues 47–67.<sup>36</sup> Furthermore, Tat expression in HeLa cells has been shown to lead to a significant increase in the nuclear levels of C/EBP $\beta$  and a corresponding increase in C/EBP $\beta$  DNA-binding activity to the IL-6 promoter.<sup>36</sup> More recently, co-expression of Tat and C/EBP $\beta$  has been shown to enhance C/EBP $\beta$  binding to the HIV-1 LTR<sup>19</sup> and modulate monocyte chemoattractant protein 1 (MCP-1) gene transcription in astrocytes.<sup>37</sup> Based on the critical role of MCP-1 in monocytic infiltration to the site of injury or inflammation in the brain<sup>38–40</sup> and the ability of C/EBP $\beta$  to stimulate the basal and Tat-mediated MCP-1 transcription, it has been proposed that the interaction between Tat and C/EBP $\beta$  may be important in HIV-1 infection, especially in the development of HAD.<sup>37</sup>

Recent studies have shown that the C/EBP-DS3 was able to regulate HIV-1 basal transcription level in U-937 cells, and the HIV-1 LTR containing a DS3 knockout phenotype (DS3-9C) exhibited a reduced level of HIV-1 basal transcription.<sup>5</sup> Other transcription factor binding sites identified downstream of the HIV-1 transcriptional start sites, including binding sites for AP-1 (I, II, III), AP3-like (AP3-L), and Sp1, have been shown to regulate HIV-1 transcription and replication.<sup>41–44</sup> Given the importance of the upstream C/EBP binding sites in HIV-1 replication in cells of the monocyte-macrophage lineage, the function of this newly identified downstream C/EBP binding site (C/EBP-DS3) was examined in this study. The studies reported herein indicate that the downstream C/EBP binding site was a functional C/EBP binding site. Transactivation of the HIV-1 LTR by C/EBP $\beta$  was inhibited when the LTR contained the DS3-9C variant. When C/EBP $\beta$  and Tat were expressed at increased concentrations, the response of the LTR depended on the concentration of each protein. If there were limited Tat and increasing levels of C/EBP $\beta$ , then loss of binding to DS3 due to the 9C variant demonstrated a decreased ability to transactivate the LTR. However, when C/EBP $\beta$  was limited with increasing amounts of Tat, the loss of binding at DS3 due to the 9C variation demonstrated an increased ability to transactivate the LTR. HIV-1 replication in U-937 monocytic cells showed a delay in replication at early time points most likely due to low levels of C/EBP $\beta$  and Tat and recovery of virus replication toward levels generated by the parental virus at later time points when there would be increased levels of Tat. Overall, DS3 plays a critical role in initiating HIV-1 subtype B transcription and replication.

## Materials and methods

### *Cell culture and cell treatments*

The U-937 human monocytic cell line (American Type Culture Collection, CRL-1593.2) was grown in Roswell Park Memorial Institute medium (RPMI)-1640 media (Cellgro). Media was supplemented with 10% heat-inactivated fetal

bovine serum (FBS; Hyclone), antibiotics, (penicillin, 100 U/mL, and streptomycin, 100  $\mu$ g/mL; Cellgro), glucose (4.5 g/L, Cellgro), sodium pyruvate (1 mM, Cellgro), and HEPES (10 mM, Cellgro). 293T cells were maintained in Dulbecco's Modified Eagle Medium (ATCC) supplemented with FBS (10%), glucose solution (10%), sodium bicarbonate (2%), and antibiotics (penicillin and streptomycin at 40  $\mu$ g/mL each). The cells were maintained at 37°C with 5% CO<sub>2</sub>.

### *Cloning and site-directed mutagenesis*

The LTR-containing DNA fragment (approximately 640 bp) was derived from the LAI molecular clone of HIV-1. The HIV-1 LAI-LTR was PCR amplified using the forward primer: 5'-GGGGTACCTGGAAGGGCTAATTCCTCC-3' and reverse primer: 5'-TCCCCCGGGTG TAGAGATTTTCCACA-3' (Integrated DNA Technologies). The italicized nucleotides indicate the restriction endonuclease binding sites used for cloning. The amplified product was digested with KpnI and Sma I (Promega, Madison, WI) and ligated into a modified pGL3-Basic vector, which contains the firefly luciferase (Luc) gene (Promega), to construct the parental LAI-LTR-Luc expression construct. The parental construct was used as a template for site-directed mutagenesis using the QuikChange mutagenesis procedure as described by the manufacturer (Stratagene) to construct the mutant construct LAI-DS3-9C-Luc. The following primers were used for site-directed mutagenesis, and the nucleotide that was mutated is underlined TAGTCAGTGTGCAAATCTCTAGC (Integrated DNA Technologies). The LAI-DS3-9C-Luc has been shown to contain the DS3 element with a G-to-C bp change at position 9 of the binding site of the subtype B consensus sequence (a sequence alteration specifically shown to completely abrogate C/EBP $\beta$  binding).<sup>5</sup> All plasmids used in these studies were sequenced to verify the sequence configurations. Sequences were analyzed using Lasergene software (DNASTAR, Inc.).

The C/EBP $\beta$ -2 expression construct was generated by PCR amplification from human C/EBP $\beta$  cDNA (Open Biosystems, Human Verified Full-length cDNA Clones, MHS 1011) utilizing forward primer: 5'-CACCATGGGAAGTGGCCAACTTCTACTA-3' and the reverse primer: 5'-CTAGCAGTGGCCGGAGGAGGCGAG-3' (Integrated DNA Technologies, Coralville, IA). The italicized nucleotides in the forward primers correspond to sequence necessary for directional cloning into the pcDNA3.1 TOPO vector (Invitrogen), while the underlined portion corresponds to the respective start site of translation. The amplified C/EBP $\beta$  PCR product was ligated into the pcDNA3.1 TOPO vector as described by the manufacturer (Invitrogen). The plasmid was verified by sequencing. To confirm proper protein expression, 30  $\mu$ g of the C/EBP $\beta$  construct was transiently transfected into 3.0  $\times$  10<sup>7</sup> 293 F cells using 293fectin as described by the manufacturer (Invitrogen), and the cell nuclear extract was harvested for further analyses

48 hours posttransfection. Western immunoblot analysis was performed with the nuclear extract using antibody specific for C/EBP $\beta$  (C/EBP $\beta$ , sc-150, Santa Cruz Biotechnology Inc.) for detection of a 45-kD protein. Electrophoretic mobility shift supershift analyses were performed to identify the specific proteins involved in DNA–protein complex formation (data not shown).

#### *Transient transfection analyses*

Exponentially growing U-937 cells were seeded at  $1 \times 10^6$  cells in 2 mL of growth medium in 6-well plates on the day of transfection. Fugene6 transfection reagent was utilized in the transient transfection as described by the manufacturer (Rocher). Briefly, 1  $\mu$ g LAI-LTR-luciferase (LAI-LTR-Luc reporter construct or LAI-LTR containing the DS3-9C variant (LTR-DS3-9C-Luc) and 50 ng pRL-TK *Renilla* luciferase internal control (Promega) were transfected together or cotransfected with other expression vectors: pcDNA3.1-C/EBP $\beta$ -2 and/or pcDNA3-Tat86. pcDNA3.1-C/EBP $\beta$ -2 is described above. pcDNA3-Tat86 expression vector was provided by Dr. Kamel Khalili (Temple University, Philadelphia, PA). The pcDNA3.1 vector without an insert was used to give each transfection an equal amount of total DNA. Cells were harvested 24 hours posttransfection, and cell lysates were prepared using 50  $\mu$ L  $1 \times$  passive lysis buffer (Promega). Luciferase activity was assayed using the dual luciferase assay system as described by the manufacturer (Promega). Normalization to an internal control plasmid was not performed in the experiments with cotransfection expression vectors because previous studies and our results have demonstrated the responsiveness of widely used internal control vectors to cotransfected transcriptional regulators.<sup>45–48</sup> Each value represents the average of triplicate transfection reactions and is representative of at least three independent experiments. The error bars shown in each figure indicate the standard deviation.

#### *Molecular clones and infection experiment*

An infectious molecular clone corresponding to the LAI strain of HIV-1 (pLAI.2) was obtained as a glycerol stock from the NIH AIDS Research and Reference Reagent Program (Catalog number 2532, NIH, MD). *Escherichia coli* containing the molecular clone were grown in Luria-broth (MILLER) supplemented with ampicillin (100 mg/mL) at 30°C, 200 RPM overnight. DNA was isolated using an EndoFree Maxiprep procedure as described by the manufacturer (USB). The 3' LTR was digested from the molecular clone using AatII and BamHI (NEBiolabs) and ligated into pUC19 (NEBiolabs). The LTR was subjected to site-directed mutagenesis to incorporate 9C mutations into C/EBP-DS3. Mutagenesis primers were the same as utilized in site-directed

mutagenesis for constructing LAI-LTR-DS3-9C-Luc described above. The mutated LTR was digested from pUC19 and ligated back into the parental molecular clone. The parental and mutant molecular clones were sequenced completely to confirm the presence of DS3 mutant and the absence of any other mutations in the HIV-1 genome subsequent to the mutagenesis process.

Molecular clone DNA (10  $\mu$ g) was transfected into 293T cells in 10 cm dishes using the Profection mammalian transfection system (E1200; Promega). Forty-eight hours after transfection, cell supernatants were collected and assayed for p24 using Enzyme-linked immunosorbent assay (ELISA) as directed by the manufacturer (Perkin Elmer). U-937 cells were seeded at a density of  $6 \times 10^4$  cells/well in a 96-well v-bottom plate. Cells were then incubated for two hours with 25 ng/mL p24 of molecular clone-derived HIV-1 LAI parental or LAI 9C strains complexed with Transfectam (Promega). Virus–Transfectam complexes were prepared by mixing 25 ng/mL p24 of virus with 5 mg/mL of Transfectam in a total of 0.5 mL of serum-free RPMI. After incubating at 37°C for 45 minutes, the medium volume was increased to 3 mL with RPMI containing antibiotics and FBS to bring the serum concentration to 10%. Following the two-hour incubation with virus, cells were washed and subsequently cultured. The supernatant was collected, and the cells were washed, supplied with new media, and split at 3-day intervals for a total of 12 days. The supernatant from days 3, 6, 9, and 12 was subsequently assayed for HIV-1 production by determining the level of p24 core antigen in the supernatant using an HIV-1 p24 antigen ELISA assay (ZeptoMetrix Corp.). Infectivity was expressed relative to mock-infected cells.

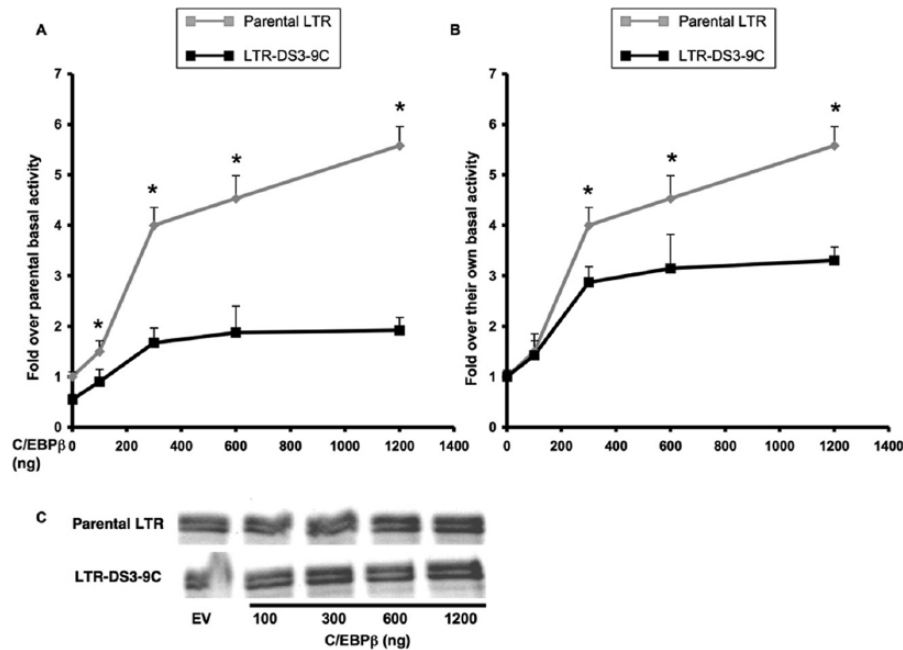
#### *Statistical analysis*

The results were statistically analyzed by Student's *t*-test. Differences between groups were considered significant if  $P < 0.05$  was obtained.

## **Results**

### *C/EBP-DS3 affects HIV-1 LTR transcription activated by C/EBP $\beta$ in U-937 cells*

Although U-937 cells represent a promonocytic cell line, undifferentiated U-937 cells are exclusively susceptible to infection by CXCR4-utilizing (X4) HIV-1 strains and have been utilized in a number of HIV-1 replication studies to examine selected aspects of the viral life cycle.<sup>49</sup> In particular, U-937 cells and X4 HIV-1 strains have been previously utilized for identifying upstream C/EBP binding sites required for HIV-1 replication in cells of the monocyte–macrophage lineage.<sup>6,7</sup> Moreover, electrophoretic mobility shift analyses have shown that HIV-1 C/EBP-DS3 is able to form a



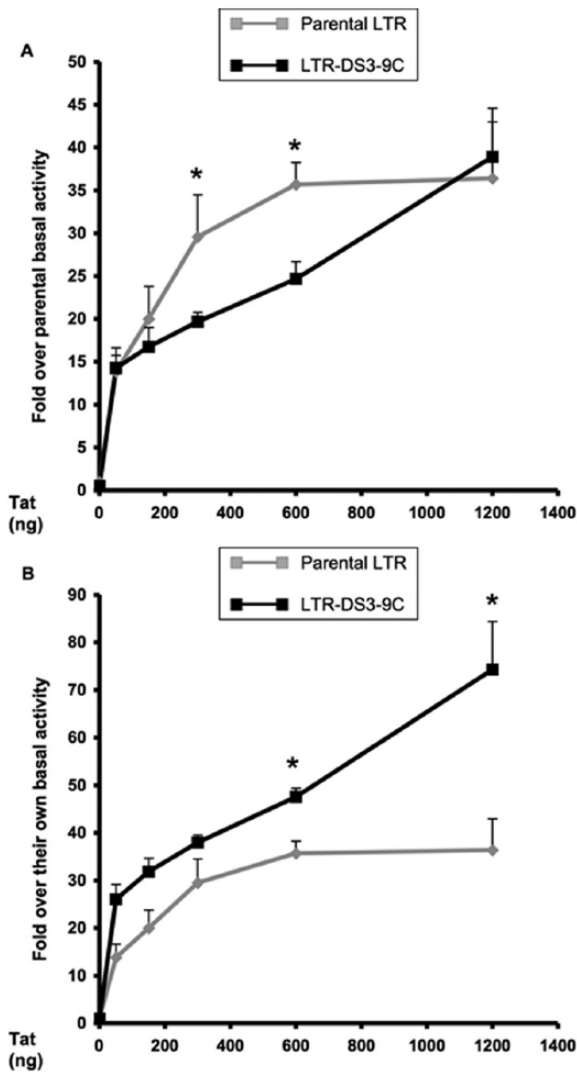
**Figure 1.** DS3-9C exhibited reduced ability to be transactivated by C/EBP $\beta$ . U-937 cells were transiently transfected with 1  $\mu$ g LAI-LTR-Luc or LAI-DS3-9C-Luc in the absence or presence of increasing amounts of C/EBP $\beta$ , as indicated in the figures. Twenty-four hours posttransfection, cell lysates were harvested and luciferase activity was measured. (A) Results were analyzed by comparing the fold over the parental basal LTR activity level. (B) Results were analyzed by comparing the fold over its own basal LTR activity level. Asterisk indicates that  $P$  value < 0.001. (C) The protein expression levels of C/EBP $\beta$  as demonstrated by Western blot were increased with the increasing amounts of C/EBP $\beta$  expression vectors cotransfected with LAI-LTR-Luc (upper) or LTR-DS3-9C-Luc (lower) in U-937 cells.

DNA-protein complex containing C/EBP $\beta$  protein from U-937 cell nuclear extract.<sup>5</sup> Therefore, the role of C/EBP-DS3 in HIV-1 transcription was first investigated in U-937 promonocytic cells.

U-937 promonocytic cells were transfected with 1  $\mu$ g parental HIV-1 LAI-LTR-Luc or the LAI-DS3-9C-Luc in the absence or presence of increasing amounts of C/EBP $\beta$ -2 (activator) expression plasmid (Figure 1). Western immunoblot assays were performed to demonstrate that the protein expression levels of C/EBP $\beta$  were increased in a dose-dependent manner when cotransfected with parental LTR or LTR-DS3-9C (Figure 1C). Expression of increasing amounts of C/EBP $\beta$ -2 (from 150, 300, 600 to 1200 ng per reaction) led to a dose-dependent increase in HIV-1 LTR activity in both the parental and DS3-9C LTR, with the parental LTR demonstrating a 2- to 5-fold higher level of activity (Figure 1). Specifically, in the presence of 1200 ng of C/EBP $\beta$  expression vector, when comparing to fold over the parental LTR basal activity, the maximal transactivation levels of parental LTR and LTR-DS3-9C were 5.5- and 1.9-fold, respectively (Figure 1A). When the results were analyzed as fold over their own basal activity, the transactivation levels were 5.5- and 3.3-fold, respectively (Figure 1B). These results indicated that, similar to the upstream C/EBP binding sites, C/EBP-DS3 was able to affect HIV-1 transcription through interaction with C/EBP $\beta$ . Specifically, the reduced occupancy of DS3 resulted in reduced ability to be transactivated by C/EBP $\beta$ .

#### *Tat activates the LTR-DS3-9C variant in U-937 monocytic cells*

Previous studies have shown that some transcription factor binding sites located downstream of the HIV-1 transcriptional start site play an important role in the Tat-mediated transactivation of the HIV-1 LTR.<sup>41,50</sup> To determine whether C/EBP-DS3 could also affect Tat transactivation of the LTR, U-937 cells were transfected with 1  $\mu$ g parental LAI-LTR-Luc or LTR-DS3-9C-Luc reporter vectors in the absence or presence of increasing amounts of Tat. As shown in Figure 2, Tat expression increased the activity of both the parental and DS3-9C-containing LTRs. In the presence of 50 ng of Tat expression vector, the activity of the HIV-1 LTR was similar between the parental and DS3-9C-containing configurations, with elevated activities of 13.8- and 14.2-fold, respectively. In the presence of increasing amounts of Tat expression vectors from 150, 300 to 600 ng, both LTRs exhibited increased activity in a dose-dependent manner. Specifically, the parental LTR transactivation levels were increased up to 20-, 29.6-, and 35.7-fold, respectively, which was higher than the levels of LTR-DS3-9C, 16.7-, 19.7-, and 24.7-fold, respectively (Figure 2A). However, when 1200 ng Tat expression vector was added to the cells, the transactivation level of LTR-DS3-9C (38.9-fold) was similar to that of parental level (36.4-fold). These results indicated that the LTR-DS3-9C variant needed much higher concentrations of Tat to be activated to levels similar to parental LTRs when there were low levels of C/EBP $\beta$  present (similar to those detected in U-937 cells). When analyzing the results



**Figure 2.** DS3-9C impacts Tat transactivation of the HIV-1 LTR. U-937 cells were transiently transfected with 1  $\mu$ g LAI-LTR-Luc or LAI-DS3-9C-Luc in the absence or presence of increasing amounts of Tat expression vectors as indicated. Cell lysates were collected and processed for luciferase activity 24 hours posttransfection. (A) Fold over parental basal activity. (B) Fold over their own basal activity. Asterisk indicates that  $P$  value < 0.05.

obtained with the parental and LTR-DS3-9C variant by comparing each LTR to its own basal transcription level, increasing Tat expression increased the transcription levels of DS3-9C-containing LTRs by 26.0-, 31.9-, 38.0-, 47.5-, and 74.3-fold, respectively, which were actually higher than the levels of the parental LTR, 13.8-, 20.0-, 29.6-, 35.7-, and 36.4-fold, respectively (Figure 2B). These results suggest that Tat can help overcome the loss of C/EBP-mediated LTR activation, at least under selected physiological conditions.

#### *HIV-1 LTR activities are further elevated by C/EBP $\beta$ and Tat together*

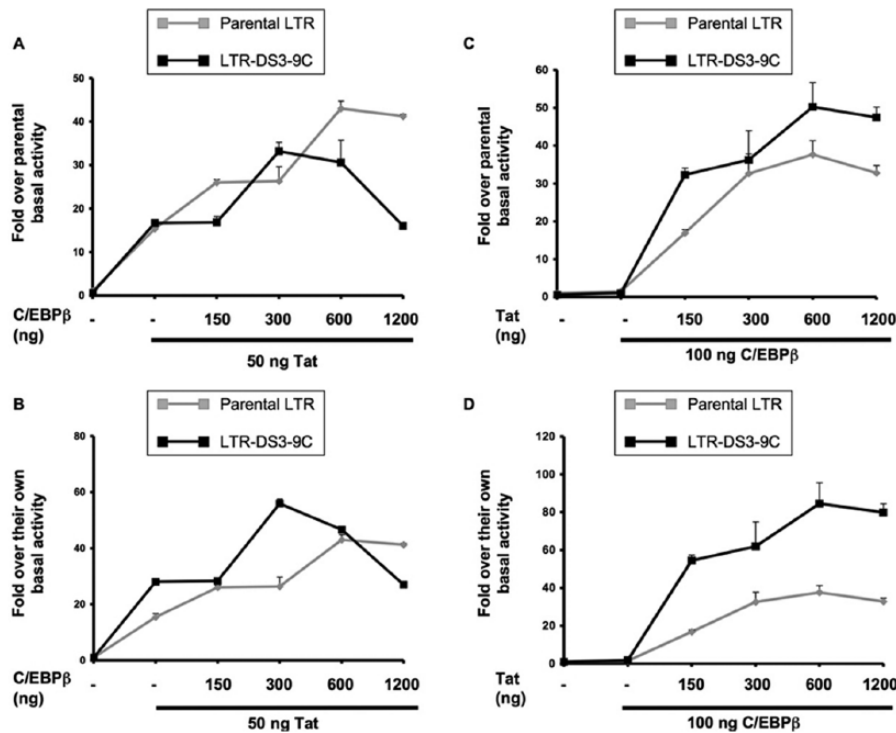
Since the LTR-DS3-9C variant exhibited a lower transactivation capability in the presence of C/EBP $\beta$  while achieving

normal or even a higher level in the presence of Tat, and both factors have been shown to be important for HIV-1 gene expression in cells of monocyte-macrophage lineage, further investigations to determine whether there was a specific interaction between C/EBP $\beta$ , Tat, and the HIV-1 LTR-DS3 variant were performed. U-937 cells were transfected with 1  $\mu$ g parental or LTR-DS3-9C in the absence or presence of 50 ng Tat expression vector and increasing amounts of C/EBP $\beta$  expression vector. As shown in Figure 3, the cooperative interaction between Tat and C/EBP $\beta$ -2 has been observed in both LTR configurations. In particular, when analyzed as fold over the parental basal LTR activity level (Figure 3A), increasing amounts of C/EBP $\beta$ -2 (100–1200 ng) increased the parental LTR from 26-, 26.3-, 43.0- to 41.3-fold, respectively, while the LTR activity of the LTR-DS3-9C variant were increased by 16.8-, 33.2-, 30.6-, and 16.0-fold, respectively. Unlike the parental LTR, with greater amounts of C/EBP $\beta$ -2 (600 and 1200 ng), the transcription level of LTR-DS3-9C was decreased. When analyzed as fold was over their own basal transcription activity (Figure 3B), the maximal transcription level of LTR-DS3-9C-driven transcription was obtained with 50 ng Tat and 300 ng C/EBP $\beta$ -2 (55.9-fold), which was higher than the highest level achieved with the parental LTR (43.0-fold). These results indicated that the LTR-DS3-9C variant exhibited a greater ability to be induced by Tat and C/EBP $\beta$ -2 together, once LTR-DS3-9C basal level was enhanced to the level of the parental LTR.

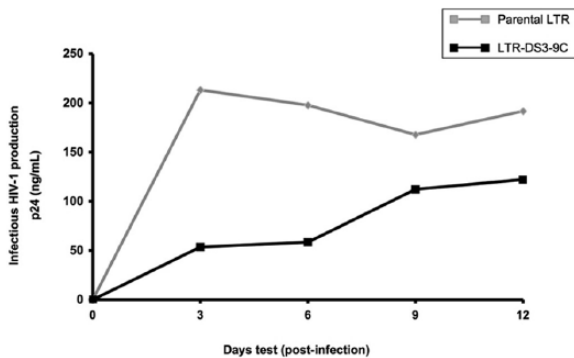
In contrast, when U-937 cells were transfected with 1  $\mu$ g parental LAI-LTR-Luc or LAI-DS3-9C-Luc in the absence or presence of 100 ng C/EBP $\beta$  expression vector and a series of increasing amounts of Tat expression vectors, LTR activity was increased in both parental and DS3-9C-containing LTRs. The maximum activity of the parental LTR and the LTR-DS3-9C variant occurred with 100 ng C/EBP $\beta$  and 600 ng Tat expression vector, 37.7- and 50.2-fold, respectively. In addition, in all Tat levels examined, LTRs containing the DS3-9C configuration resulted in increased LTR activity over the parental LTR demonstrating that loss of C/EBP binding to DS3 can be overcome by increased expression of C/EBP $\beta$  (which can be found in the cells of the activated monocyte-macrophage lineage) and increased expression of Tat, leading one to conclude that DS3-9C may be involved in controlling HIV-1 replication in activated monocyte-macrophage environments.

#### *DS3-9C decreases HIV-1 LAI replication in U-937 monocytic cells*

To determine the effects of DS3 on HIV-1 replication, the DS3-9C variation was incorporated into an infectious molecular clone of the LAI strain of HIV-1. Molecular clone-derived viral particles were used to infect U-937 cell lines (Figure 4). Levels of p24 in the media were measured at 3, 6, 9, and 12 days postinfection. In U-937 cells, when compared with the parental LAI viral strain, the HIV-1 DS3-9C-containing



**Figure 3.** Tat and C/EBP $\beta$  were able to cooperatively increase HIV-1 parental LTR and LTR-DS3-9C. (A) and (B) U-937 cells were transiently transfected with 1  $\mu$ g LAI-LTR-Luc or LTR-DS3-9C-Luc in the absence or presence of 50 ng Tat and increasing amounts of C/EBP $\beta$  (100, 300, 600, to 1200 ng) together. (C) and (D) U-937 cells were transiently transfected with 1  $\mu$ g LAI-LTR-Luc or LAI-DS3-9C-Luc in the absence or presence of 100 ng C/EBP $\beta$  and increasing amounts of Tat (100, 300, 600, to 1200 ng) together. Twenty-four hours posttransfection, cell lysates were collected and luciferase activity was measured. The results shown in (A) and (C) indicate the fold over parental LTR basal activity, and the results shown in (B) and (D) indicate the fold over their own basal transcription level.



**Figure 4.** HIV-1 LAI molecular clones containing DS3-9C demonstrated an altered replication phenotype. Molecular clone-derived viral particles were used to infect U-937 monocytic cells as described in the “Materials and methods” section. Levels of p24 in the media were measured at 3, 6, 9, and 12 days postinfection. The gray line corresponds to the HIV-1 LAI parental virus. The black line corresponds to the LAI virus containing the DS3-9C variant configuration.

variant viruses resulted in lower replication levels at each time point examined. However, the maximal p24 level of parental virus was 213 ng/mL at 3 days postinfection, after which time lower levels of virus were observed. However, the replication levels of HIV-1 DS3-9C variant viruses continuously increased from day 3 (p24 level was 53 ng/mL) to day 12 (p24 level was 112 ng/mL) postinfection (Figure 4). Although the HIV-1

LAI strain, a CXCR4-utilizing virus, was examined in this study, it was able to effectively infect U-937 cells, which has also been demonstrated in other studies.<sup>51,52</sup> Therefore, the comparison of the replication levels of these two viruses indicated that the DS3-9C variant exhibited a decreased replication ability at early time points but clearly has the ability to reach parental levels at later time points.

## Discussion

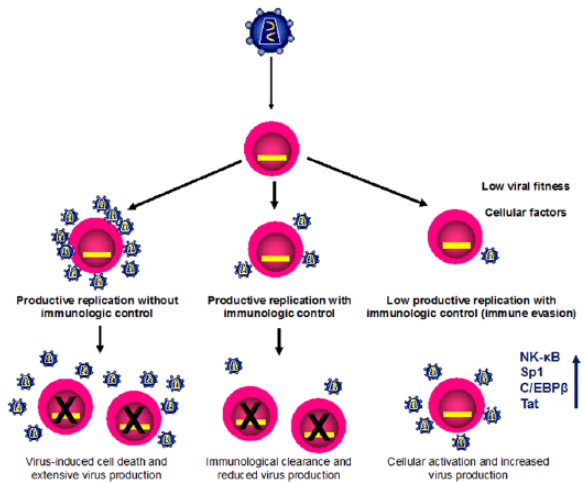
Although numerous studies have investigated the important roles played by upstream *cis*-acting transcription factor binding sites and their cognate transcription factors regulating HIV-1 gene expression, relatively few studies have been reported concerning the transcription factor binding sites located downstream of the transcriptional start site of HIV-1 LTR. The sequence analyses have shown that some transcription factor binding sites located downstream of the transcriptional start site exhibit a high degree of sequence conservation by comparison to the subtype B consensus sequence.<sup>5</sup> High sequence conservation may indicate the positive selection of these sites during the course of viral evolution within patients and across the infected patient population. For example, the downstream AP-1 binding sites within subtype B LTRs have been shown to be highly conserved genotypically<sup>5</sup> and affect the basal and Tat transactivation ability of the HIV-1 LTR.<sup>42,50</sup> Proviruses



containing mutations in three AP-1 binding sites abolished HIV-1 replication in peripheral blood mononuclear cells and T-lymphocyte cell lines.<sup>41</sup> Therefore, to better understand the pathogenesis of HIV-1, a more thorough characterization of the HIV-1 LTR is required, including studies of the transcription factor binding sites located downstream of the transcriptional start site.

Cells of the monocyte–macrophage lineage are important for HIV-1 replication and long-term persistence of HIV-1. Unlike T-lymphocytes, cells of the monocyte–macrophage lineage infected with HIV-1 are resistant to the cytopathic effects of the virus and serve as a long-lived reservoir for HIV-1-persistent infection.<sup>49,53</sup> Furthermore, the ability of macrophages to migrate into other tissues or to invade the brain is relative to a number of HIV-1-associated diseases, including HAD.<sup>54</sup> Additionally, studies have shown that cells of monocytic origin are able to harbor latent HIV-1 provirus in all stages of the disease even in patients receiving successful highly active antiretroviral therapy, indicating that in addition to resting CD4+ T-cells, monocyte–macrophage are another potential latent virus reservoir, which may be able to continue to accumulate and harbor replication-competent HIV-1.<sup>55–58</sup> Results reported herein suggest that one downstream C/EBP site with low DNA binding affinity for C/EBP $\beta$  (DS3-9C) might be related to HIV-1 persistence and reactivation in cells of the monocyte–macrophage lineage.

HIV-1 LTRs containing the DS3-9C configuration (a knockout configuration) exhibit relatively lower basal<sup>5</sup> and C/EBP $\beta$ -mediated LTR activity (Figure 1), suggesting that DS3-9C could function as a negative regulatory element, to suppress HIV-1 transcription, especially at the beginning of infection when minimal viral protein has been produced yet, helping HIV-1 to evade the immune response by essentially increasing the energy of activation required to achieve the productive virus replication phase driven by high concentrations of Tat. However, once the infected cells are activated by proper extracellular stimuli or in the presence of enough Tat protein, LTR-DS3-9C could function at levels comparable to parental LTRs or even with higher LTR activity (Figures 2 and 3). Specifically, compared with parental LTR, although the activity levels of LTR-DS3-9C were lower at certain quantities of Tat (150, 300, and 600 ng), LTR-DS3-9C was able to be activated to the similar levels in the presence of 50 and 1200 ng Tat, respectively, indicating that (1) LTR-DS3-9C was able to respond rapidly to the low quantities of Tat, which could be important for HIV-1 transcription initiation, and (2) LTR-DS3-9C was able to act as transcription-competent LTR in the presence of large quantities of Tat. The results also suggested that once LTR-DS3-9C activation was initiated, which was approximated in the assays by analyzing the results as fold over their own basal level, the maximal activity level of LTR-DS3-9C activation by Tat (74.3-fold) was significantly higher than that of parental LTR (36.6-fold), suggesting that (3)



**Figure 5.** Three possible outcomes of HIV-1 infection in T-lymphocytes and cells of the monocyte–macrophage lineage. Left: productive HIV-1 infection without immunologic control; middle: productive infection with immunologic control; right: low productive infection with immune evasion. Since the infected cells are activated, the expression of cellular factors (NF- $\kappa$ B, Sp1, and C/EBP $\beta$ ) are increased and/or enough amounts of Tat are available, these viruses are able to be transcription and replication competent.

LTR-DS3-9C exhibited a greater degree of Tat inducibility. Additionally, when the LTR-DS3-9C was activated by the small amounts of C/EBP $\beta$ , LTR-DS3-9C exhibited a higher level of transactivation in the presence of Tat (Figure 3C and D), which further confirmed LTR-DS3-9C as a transcription-competent variant. The replication results demonstrated that although the levels of DS3-9C variant viruses were lower than those of the parental virus during the first 12 days postinfection, the levels were continuously increased while those of parental viruses attained a maximal level at 3 days postinfection and then started to decline (Figure 4), suggesting that there may be a delay in virus replication. Taken together, these results suggest that this site is regulated by the cell activation state and produced viral proteins, especially, the amounts of C/EBP $\beta$  and Tat present within the infected cell.

HIV-1 infection in T-lymphocytes or cells of the monocyte–macrophages could result in three possible outcomes: (1) productive replication without efficient immune recognition leading to extensive viral production and the death of the host cells; (2) productive replication with immune recognition leading to the clearance of infected cells prior to high-level virus production; and (3) infection leading to limited viral gene expression and failure to eliminate infected cells by the immune system with continued maintenance of latent or persistent provirus within the infected cell population (Figure 5). Based on these observations, we propose that infection with virus containing the LTR-DS3-9C variant could lead to the third outcome. Specifically, during the early stage of HIV-1-DS3-9C infection, no Tat protein was produced and the transcription was totally dependent on host cell factors (NF- $\kappa$ B, Sp1,

C/EBP $\beta$ , CBP/p300, PCAF, etc.).<sup>17,59–62</sup> LTR-DS3-9C-containing viruses exhibited relatively low basal transcription and replication level, which helped DS3-9C viruses evade immune system surveillance and perhaps be retained in the infected cell population. With disease progression and/or some external stimuli, the infected cells were activated; the active form of NF- $\kappa$ B could be produced and translocated to the nucleus; and the expression levels of Sp1 and C/EBP $\beta$  were increased, all promoting HIV-1 transcription initiation. Subsequently, Tat was produced, and efficient transcription elongation occurred, allowing DS3-9C variant viruses to replicate with a delayed phenotype.

Recent studies have shown the linkage between HIV-1 replication and disease progression with genetic alterations in selected transcription factor binding sites within the HIV-1 LTR, such as Sp1-binding sites<sup>63,64</sup> and US1 C/EBP site.<sup>10</sup> It is possible that virus containing the DS3-9C variant might be associated with a greater propensity to establish latency. Defective HIV-1 transcription, which could be caused by (1) low levels of NF- $\kappa$ B,<sup>65–67</sup> (2) low levels of Tat,<sup>68–72</sup> and (3) limited cellular coactivators, such as cyclin T1, a component of the p-TEF complex,<sup>65,73</sup> is one of the major reasons for HIV-1 latency. Although transient transfection provides a simpler chromatin structure, it helps in understanding activities of integrated LTRs. The establishment of an open nuc-1 is critical for HIV-1 gene expression<sup>74,75</sup> and DS3-9C is located at the 3' edge of nucleosome-1 (nuc-1), so it is possible that DS3-9C variant may lead to a structural change in the LTR that results in a specific restrictive chromatin structure limiting the accessibility of Tat and coactivators (HAT and/or SWI/SNF) to the LTR, thereby resulting in a low level of HIV-1 transcription and possibly latency. Within the context of proper stimuli, the latent viruses are able to reactivate and function as parental viruses.

## Conclusion

The function of one C/EBP binding site located downstream of the HIV-1 LTR transcription start site has been characterized. This binding site configuration with low DNA binding affinities for C/EBP $\beta$  (DS3-9C) may be transcriptionally competent and be able to facilitate productive replication in the presence of Tat. This LTR variant may promote HIV-1 persistence and reactivation in cells of the monocyte-macrophage lineage. Further experiments will examine the role of this interesting *cis*-acting element *in vivo*, utilizing stably integrated LTRs or genomes containing the DS3-9C variant in different cell types under an assortment of stimulatory conditions. With respect to the important roles played by upstream and downstream C/EBP binding sites in the regulation of HIV-1 gene expression in cells of the monocyte-macrophage lineage, C/EBP binding sites may be the potential targets for design of novel forms of HIV-1 therapeutics.

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## Author contributions

Conceived and designed the experiments: YL, MRN, AA, VP, AB, LL, EK, and BW. Analyzed the data: YL, MRN, VP, AB, EK, and BW. Wrote the first draft of the manuscript: YL. Contributed to the writing of the manuscript: YL, MRN, AA, VP, AB, LL, EK, and BW. Agreed with manuscript results and conclusions: YL, MRN, AA, VP, AB, LL, EK, and BW. Jointly developed the structure and arguments for the paper: YL, MRN, and BW. Made critical revisions and approved the final version: YL, MRN, AA, VP, AB, LL, EK, and BW. All the authors reviewed and approved the final manuscript.

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