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Structural and functional studies of CCAAT/enhancer binding sites within the human immunodeficiency type 1 subtype C LTR

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Abstract

Human immunodeficiency virus type 1 (HIV-1) subtype C, which is most predominant in sub-Saharan Africa as well as in Asia and India, is the most prevalent subtype worldwide. A large number of transcription factor families have been shown to be involved in regulating HIV-1 gene expression in T lymphocytes and cells of the monocyte-macrophage lineage. Among these, proteins of the CCAAT/enhancer binding protein (C/EBP) family are of particular importance in regulating HIV-1 gene expression within cells of the monocytic lineage during the course of hematologic development and cellular activation. Few studies have examined the role of C/EBPs in long terminal repeat (LTR)-directed viral gene expression of HIV-1 subtypes other than subtype B. Within subtype B viruses, two functional C/EBP sites located upstream of the TATA box are required for efficient viral replication in cells of the monocyte-macrophage lineage. We report the identification of three putative subtype C C/EBP sites, upstream site 1 and 2 (C-US1 and C-US2) and downstream site 1 (C-DS1). C-US1 and C-DS1 were shown to form specific DNA-protein complexes with members of the C/EBP family (C/EBP α , β , and δ). Functionally, within the U-937 monocytic cell line, subtype B and C LTRs were shown to be equally responsive to C/EBP β -2, although the basal activity of subtype C LTRs appeared to be higher. Furthermore, the synergistic interaction between C/EBP β -2 and Tat with the subtype C LTR was also observed in U-937 cells as previously demonstrated with the subtype B LTR.

Keywords

C/EBP β ; HIV-1; Subtype C; LTR; Transcription

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is divided into three groups, M (major), O (outlier), and N (non-M, non-O). Most HIV-1 isolates belong to the M group, which has been subdivided into at least nine subtypes: A–D, F–H, J–K, and several circulating recombinant forms (CRFs) [1–3]. Most experimental studies concerning HIV-1 center on subtype B, the predominant form in North America and Europe, which accounts for approximately 10% of HIV-1 infections worldwide. In contrast, subtype C, which causes more than 50% of HIV-1 infections and predominates in southern Africa, India, and other parts of Asia, has been under investigated [4,5].

Several studies have demonstrated that different HIV-1 subtypes have distinct long terminal repeat (LTR) structures that represent the promoter sequences that drive HIV-1 gene expression and have been shown to influence viral transcription and replication in a cell type-specific manner [6–8][9]. For example, several studies have demonstrated the presence of three nuclear factor (NF)- κ B binding sites within the subtype C LTR, whereas most other subtypes contain two NF- κ B binding sites. One NF- κ B binding site has been identified in CRF01_AE [10,11]. The extra NF- κ B site in the subtype C LTR has been shown to be related to the high level of basal transcription and increased tumor necrosis factor α -induced transcriptional activity [10,12,13]. Three CCAAT/enhancer binding protein (C/EBP) sites (B-US1, B-US2, and B-US3) located upstream of the transcriptional start site have been identified within the subtype B LTR [14]. Activation of one of the C/EBP binding sites (B-US1 or B-US2) through binding of C/EBP factors is required for HIV-1 subtype B transcription and replication in cells of the monocyte-macrophage lineage [15,16]. Studies have shown, however, that C/EBP B-US2, which is present in the –170 region of the HIV-1 subtype B LTR, is not present in the subtype C LTR [17]. No other information has been reported regarding the existence or function of C/EBP sites within the subtype C LTR.

The C/EBP family of proteins belong to the basic region leucine zipper transcription factor family, which includes at least six members: C/EBP α , β , γ , δ , ϵ , and ζ [18]. C/EBP family members, especially C/EBP β , have been reported to regulate HIV-1 transcription within different cell types (monocyte-macrophages, T cells, glial and microglia cells, endothelial cells, and hepatocytes) through various mechanisms [15,16,19–25], including interacting with several other transcription factors that include NF- κ B [26,27], Sp1 [20], activating transcription factor/cyclic adenosine monophosphate-responsive element binding protein (ATF/CREB) [28–30], viral proteins Vpr and Tat [31–33], and chromatin remodeling factors, CREB binding protein/p300 and SWI/SNF (switch/sucrose nonfermentable) [22,34,35]. There are three isoforms of human C/EBP β , C/EBP β -1, -2, and -3 [36]. C/EBP β -1 and -2 isoforms act as transcriptional activators and C/EBP β -3 functions as a transcriptional repressor [36].

CCR5 (R5) and CXCR4 (X4) are the predominant co-receptors for HIV-1 entry (for review see Clapham and McKnight [37]). CCR5 acts as the major co-receptor for macrophage-tropic HIV-1 strains [38], whereas T-cell line-adapted viruses use CXCR4 [39,40]. Interestingly, most subtype C viruses examined to date display a preferential usage of the CCR5 co-receptor [41–43]. Based on the role of the C/EBP transcription factor family in HIV-1 subtype B replication in cells of the monocyte-macrophage lineage and CCR5 usage appearing to be more frequent among subtype C viruses, we sought to determine whether C/EBP binding site(s) also exist in the subtype C LTR and to determine the functional properties of the C/EBP binding sites within the context of HIV-1 subtype C transcription.

The HIV-1 transactivator protein (Tat) is critical for HIV-1 transcription and regulation (reviewed in [44–47]) by interacting with the transactivation-responsive region, located at

the 5' end of all nascent HIV-1 transcripts [48,49]. Variation in viral genomic sequence has been reported, as well as the resultant amino acid changes and subtype-specific effects of Tat protein among the different HIV-1 subtypes [10,11,50–52]. Specifically, Tat proteins from subtype C and E have been shown to drive higher levels of viral gene expression than subtype B Tat regardless of the origin of the HIV-1 LTR [13,50]. The increased level of viral gene expression observed with subtype C and E is related in part to the longer half-life of Tat within infected cells [50].

The studies reported here have determined that one C/EBP binding site exists both upstream and downstream of the transcriptional start site within HIV-1 subtype C LTR. In general, the subtype C LTR exhibited a higher basal transcription level compared with the subtype B LTR under the experimental conditions examined. C/EBP β -2 was able to activate HIV-1 subtype C LTR-driven transcription in the U-937 promonocytic cell line with an overall level of transcription close to that of the subtype B LTR. A further increase in subtype C LTR activity was observed in the presence of both C/EBP β -2 and subtype C Tat.

2. Material and Methods

2.1. Cell culture and nuclear extract preparation

The U-937 human monocytic cell line (American Type Culture Collection, CRL-1593.2) was grown in RPMI-1640 (Roswell Memorial Park Institute medium) (Cellgro, Mediatech, Manassas, VA). Medium was supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μ g/ml; Cellgro), glucose (4.5 g/ml, Cellgro), sodium pyruvate (1 mM, Cellgro), and HEPES [4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid], 10 mM; Cellgro). The cells were maintained at 37°C with 5% CO₂ in 90% relative humidity.

Nuclear extracts were prepared as described [53]. Low-passage, exponentially growing cells (1×10^7) were collected by centrifugation, washed with cold phosphate-buffered saline (Cellgro), and lysed in ice-cold lysis buffer. After centrifugation ($1000 \times g$), the supernatant was discarded and the pelleted nuclei were resuspended in nuclear extract buffer, shaken vigorously for 30 min at 4°C, and subjected to centrifugation for 15 min ($14,000 \times g$). The supernatant was collected, frozen in liquid nitrogen, and stored at -80°C . The protein concentration was determined by Bradford assay as described by the manufacturer (Bio-Rad, Hercules, CA).

2.2. Electrophoretic mobility shift (EMS) analyses

Complementary single-stranded DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Probes were annealed by heating at 100°C for 5 min and slowly cooling to room temperature. Probes were 5' end-labeled using T4 DNA kinase (Promega, Madison, WI) and [γ -³²P] adenosine triphosphate. Labeled probes (75,000 cpm/lane) were incubated with 2 μ g of nonspecific DNA (poly [d(I-C)], Roche, Indianapolis, IN) and 20 μ g of U-937 nuclear extract at 30 °C for 30 min. Competition studies were performed using unlabeled competitor oligonucleotides at concentrations up to 100-fold molar excess as compared with the probe concentration. Supershift analyses were initiated by incubation of the complexes for another 15 min with antibodies specific to C/EBP α (sc-9315x), C/EBP β (sc-150x), C/EBP γ (sc-7659x), C/EBP ϵ (sc-31929x), or C/EBP δ (sc-636x) (all from Santa Cruz Biotechnologies, Santa Cruz, CA). DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels at 200 V at 4°C for approximately 2 h. Gels were dried at 80°C for 1 h and subjected to autoradiography.

2.3. Plasmids

The subtype C LTR-containing DNA fragment (approximately 630 bp) was derived from the HIV-1 subtype C molecular clone (pMJ4, National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, MD). The HIV-1 subtype C LTR was PCR-amplified using the forward primer: 5'-*GGGGTACCTGGAAGGGTTAATTTACTCT*-3' and reverse primer: 5'-*CGACGCGTTAGAG ATTTTCCTCACTACC*-3' (Integrated DNA Technologies). The italicized nucleotides indicate the restriction endonuclease sites. The amplified product was digested with *KpnI* and *MluI* (Promega) and ligated into a modified pGL3-basic vector, which contained the firefly luciferase (Luc) gene (Promega), to construct the parental HIV-1 subtype C LTR-luciferase (C-LTR-Luc) expression construct. The HIV-1 LAI LTR was cloned into the pGL3-basic vector as previously described [54].

The C/EBP β -2 expression construct was generated by PCR amplification from human C/EBP β cDNA (Open Biosystems, Human Verified Full-length cDNA Clone, MHS 1011, Huntsville, AL) using forward primers for C/EBP β -2, 5'-*CACCATGGAAGTGGCCAACCTTCTACTA*-3' and the reverse primer 5'-*CTAGCAGTGGCCGGAGGAGGCGAG*-3' (Integrated DNA Technologies). The italicized nucleotides in the forward primer correspond to the sequence necessary for directional cloning into the pcDNA3.1 TOPO vector (Invitrogen, San Diego, CA), and the underlined portion corresponds to the respective start site of translation. The amplified C/EBP β -2 PCR product was ligated into the pcDNA3.1 TOPO vector as described by the manufacturer (Invitrogen). The plasmids were verified by sequencing (Genewiz, South Plainfield, NJ). To confirm expression, 30 μ g of the C/EBP β -2 construct was transiently transfected into 3.0×10^7 293F cells using 293fectin as described by the manufacturer (Invitrogen). Forty-eight hours after transfection, nuclear extracts were collected and western immunoblot analyses were performed on nuclear extracts using C/EBP β antibody (C/EBP β , C-19, Santa Cruz Biotechnologies) for detection of a 45-kDa protein, C/EBP β -2. Electrophoretic mobility shift (EMS) supershift analyses were performed to determine the identity of proteins involved in DNA-protein complex formation [Liu and Wigdahl, unpublished observations].

2.4. Transient expression analyses

Exponentially growing U-937 cells (1×10^6) were seeded in 2 ml of growth medium in six-well plates. For each transfection, FuGENE 6 Transfection Reagent (6 μ l) (Roche) was dispensed into serum-free medium (94 μ l). After 5 min, the solution was added to the DNA dropwise, incubated for 15 min, and dispensed dropwise into the cell culture. Cells were transfected with 1000 ng firefly luciferase LTR expression constructs (C-LTR-Luc or B-LTR-Luc) in conjunction with the thymidine kinase promoter-Renilla luciferase reporter plasmid (pRL-TK) internal control vector (50 ng). Cells were harvested 24 h after the addition of the FuGENE 6 and DNA. Cell lysates were harvested and assayed using the Dual Luciferase Assay system as described by the manufacturer (Promega). Firefly luminescence was normalized to *Renilla* luminescence to control for variation in transfection efficiency. For the experiments that included C/EBP β -2 expression vectors, single luciferase assays were performed because C/EBP β -2 has been shown to activate the pRL-TK promoter [53]; Liu and Wigdahl, unpublished observations]. Cell lysates were first quantified by Bradford assays (Promega) and cell lysates (30 μ g per sample) were used for performance of single luciferase assays. The results are presented as mean values with the indicated standard deviations. Each value shown represents the average of two or three independent experiments performed in triplicate.

2.5. Statistical analysis

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

3. Results

3.1. Identification of C/EBP binding sites within the HIV-1 subtype C LTR

To determine whether the HIV-1 subtype C LTR contained C/EBP binding site(s), 211 subtype C LTR sequences were collected from GenBank and Los Alamos HIV sequence databases. The consensus sequence of the subtype C LTR was determined by aligning the 211 LTR sequences using the MegAlign program (DNASar, Madison, WI) with the Clustal W algorithm (European Bioinformatics Institute, Cambridge, UK). The LTR subtype C consensus sequence was analyzed using the Web-based program TRANSFAC (P-Match; BioBase Biological Databases, Wolfenbüttel, Germany), (0.75 core similarity, 0.85 matrix similarity threshold) for the presence of putative C/EBP binding sites. As a result of this analysis, two sites were located upstream and one site was located downstream of the transcriptional start site within the subtype C LTR. They were designated subtype C C/EBP upstream site 2 (C-US2), upstream site 1 (C-US1), and subtype C C/EBP downstream site 1 (C-DS1). C-US2 was located at nucleotide position -446 to -433; C-US1 was located at position -259 to -247 and C-DS1 was located at position +171 to +184, relative to the transcriptional start site (Fig. 1A). When compared with the three identified upstream C/EBP binding sites within subtype B LTR (B-US3, B-US2, and B-US1, Fig. 1A), the physical location and nucleotide sequences of subtype C were very different (Fig. 1A and B). In contrast, the physical location and nucleotide sequence of the C/EBP binding site located downstream of the transcriptional start site were similar between subtype B and C LTRs (Fig. 1A and B).

To determine whether the three putative C/EBP binding sites were able to form DNA-protein complexes with C/EBP protein family members, supershift EMS analyses were performed. The oligonucleotides representing the three putative subtype C consensus C/EBP binding sites were radiolabeled and used as probes (Fig. 1C) and incubated with nuclear extracts prepared from U-937 monocytic cells. Antibodies specific to C/EBP α , β , γ , ϵ , or δ were used in EMS reactions. As shown in Fig. 1C, C-US2 was unable to form specific DNA-protein complexes and was therefore eliminated from further analysis. In contrast, the DNA-protein complexes formed by C-US1 or C-DS1 were shifted by antibodies specific to C/EBP α , β , and δ (Fig. 1C). These results indicated that the subtype C C/EBP binding sites C-US1 and C-DS1 had the ability to form specific DNA-protein complexes containing C/EBP α , β , and δ .

3.2. Comparison of C/EBP binding sites and other important transcription factor binding sites between subtype B and C LTRs

The interactions between subtype B C/EBP US1 (B-US1) and transcription factor binding sites located in proximity, including ATF/CREB, NF- κ B, and Sp site III binding sites, have been reported [26,28,30]; Burdo and Wigdahl, unpublished observations]. To determine whether similar transcription factor binding sites were also located near subtype C-US1, the subtype C LTR consensus sequence was analyzed by TRANSFAC. Sequence analyses (0.75 core similarity, 0.85 matrix similarity threshold), however, failed to identify any transcription factor binding sites known to be important for HIV-1 transcription located in the 5' or 3' sequences immediately adjacent to C-US1. In contrast, the ATF/CREB site located 5' of B-US1 also existed immediately 5' of the promoter-distal NF- κ B site in the subtype C LTR with the same physical location and only two nucleotide differences (Fig. 2). Also, three Sp binding sites (Sp sites I, II, and III) were located 3' to the promoter-proximal NF- κ B binding site in both subtype B and C LTRs with the same physical location and highly conserved nucleotide sequence (Fig. 2). Compared with consensus sequences of subtype B Sp binding sites, consensus sequences of subtype C Sp binding sites exhibited similar nucleotide sequences; Sp site I contained only one nucleotide variation (C-to-T

change at position 10), and Sp site III contained three nucleotide variations (a deletion at position 1 and a C-to-T change at positions 5 and 10). With respect to the subtype C NF- κ B binding sites, the nucleotide sequences of NF- κ B site I and II were conserved between subtypes C and B (Fig. 2). The extra NF- κ B binding site (NF- κ B III), however, exhibited nucleotide sequences different from that of the subtype B or C consensus sequence.

The prevalences of the consensus sequence within the C/EBP US1 site, ATF/CREB site, NF- κ B sites, and Sp sites I, II, and III were compared between the subtype B and C LTRs. For subtype B, the sequences from 348 peripheral blood-derived LTRs documented in three published studies [55–57] were used in the analyses of C/EBP US1; Sp sites I, II, III; and NF- κ B site II. In addition, 267, 223, and 131 LTRs were used in C/EBP US2, NF- κ B site I, and ATF/CREB site analyses, respectively. For subtype C LTR analysis, among the 211 collected LTRs, 197 LTRs have been used for three NF- κ B sites, Sp sites I and II, and ATF/CREB binding sites with 205 and 185 LTRs used for C/EBP US1 site and Sp site III analyses, respectively. In the analyses in which a lower number of sequences were used, sequences for these regions were not available. Based on these analyses, C/EBP US1 exhibited similar conservation between subtype B (52.8%) and C (50.7%) (Fig. 3). Interestingly, among the subtype C transcription factor binding sites examined, the ATF/CREB site was highly conserved (93.9%); this was much higher than the subtype B ATF/CREB site (74.8%). The two classical NF- κ B binding sites and Sp site II from subtype B and C LTRs also exhibited a high degree of conservation (subtype C NF- κ B site II, I, Sp site II was 89.3%, 95.9% and 89.8%, respectively; subtype B NF- κ B site II, I, Sp site II was 96%, 91%, and 83%, respectively), which was correlated with the important roles these transcription factor binding sites play in HIV-1 transcription, whereas Sp site I was significantly less conserved (36%) in the subtype C LTR than in subtype B LTR (86.2%). Currently, it is not clear why Sp site I is not conserved in subtype C. It has been suggested that this site may be critical for subtype B gene expression. The differences noted in relative conservation warrant further investigation. Importantly, the degree of conservation of Sp site III, which is known to be a critical regulator of HIV-1 replication in both T cells and cells of the monocyte-macrophage lineage, is similar for the both the subtype B and subtype C LTRs (32.5% and 43.2%, respectively). For the C/EBP binding site located downstream of the transcriptional start site within subtype B LTR, 77 of 100 LTRs (77%) exhibited the consensus sequence. With respect to the upstream C/EBP binding site located within subtype C LTR, 66 of 115 (57%) were found to represent the consensus sequence configuration [Liu and Wigdahl, unpublished observations].

3.3. Differing number and location of C/EBP binding sites within the subtype B and C LTRs, yet similar level of response to the transactivator C/EBP β

Previous studies have demonstrated that representative subtype C LTRs exhibited higher basal transcription levels compared with several subtype B LTRs; however, most of these experiments were performed in T-cell lines and primary T lymphocytes [10,13,58,59]. Given that most isolated subtype C viruses preferentially use CCR5 as a co-receptor throughout the course of disease and therefore may rely more heavily on replication in cells of the monocyte-macrophage lineage as compared with a subtype B virus, which usually switches co-receptor usage from CCR5 to CXCR4 as HIV disease progresses, basal transcription levels of representative subtype B and C LTRs were compared in U-937 monocytic cells. Plasmids containing parental subtype B LTR (LAI) or a subtype C LTR (pMJ4) derived from a clinical isolate and obtained from the AIDS Reference and Reagent Program were transiently transfected into U-937 monocytic cells and LTR function was assessed based on the expression of the luciferase indicator gene. The representative subtype C LTR exhibited approximately 25% higher LTR basal activity than the representative subtype B LTR in U-937 monocytic cells (Fig. 4A). HIV-1 subtype C LTR transcription

levels as a result of co-transfection with the C/EBP β -2 expression vectors (Fig. 4B) were also examined in U-937 monocytic cells (Fig. 4C). C/EBP β -2 (activator) was able to transactivate HIV-1 LTR transcription (Fig. 4B). As shown in Fig. 4C, in the presence of C/EBP β -2, the transcription level of the subtype C LTR was enhanced up to 5.2-fold in U-937 cells. In parallel, the same assays were performed with the subtype B LTR. These results reveal that the level of transcription was increased up to 5.3-fold in U-937 cells. As shown, there was no significant difference with respect to C/EBP β -2 activation of either the subtype B or C LTRs, even with the different C/EBP binding site number, nucleotide sequences, and/or physical location.

3.4. Increased transactivation of subtype C LTR in the presence of Tat and C/EBP β as compared with Tat alone

The transcription level of the subtype C LTR activated by subtype C Tat was determined in U-937 monocytic cells. As expected, in the presence of a subtype C Tat expression vector, the transcription level was enhanced up to 35.5-fold. Previous studies have shown the synergistic interaction between subtype B Tat and C/EBP β -2 with respect to activating the subtype B LTR [27]. Similarly, the combined presence of subtype C Tat and C/EBP β -2 resulted in a 73.7-fold increase in subtype C LTR transcriptional activity (Fig. 5).

4. Discussion and conclusion

Previous studies have demonstrated that (1) the corresponding subtype B C/EBP US2 does not exist in the subtype C LTR [17]; (2) subtype B US1 location has been replaced by an NF- κ B site in the subtype C LTR; (3) C/EBP binding sites (US2 or US1) are required for HIV-1 subtype B transcription and replication in cells of the monocytic-macrophage lineage [15,16,25]; and (4) subtype C viruses prefer to use CCR5 as a co-receptor for virus entry throughout the course of HIV disease [41–43]. It has not been demonstrated, however, whether C/EBP binding sites exist within the HIV-1 subtype C LTR and, if they are present, whether they function within target cells representative of cells encountered by the subtype C virus during the course of disease. Our results demonstrate the presence of C/EBP binding sites within the subtype C LTR, located upstream and downstream of the transcriptional start site. Although the C/EBP binding site located upstream of the transcriptional start site within the subtype C LTR exists in a distinct location and also displays a unique spectrum of genotypic alternations as compared with the subtype B upstream functional C/EBP sites (B-US2 and -US1), both LTRs have been shown to be important for viral transcription and were shown to exhibit similar activity in the presence of C/EBP β -2, suggesting that the function of C/EBP β transactivation is conserved and important for HIV-1 transcription in different subtypes. Therefore, it would be interesting to investigate the roles of C/EBP factors and C/EBP sites in different HIV-1 subtypes. The location of C-US1 is close to that of B-US3 (Fig. 3A), which is not required for HIV-1 subtype B LTR transcription [16,19]. Thus, it is possible that the different genotype of C-US1 results in the functional C/EBP binding site or that C-DS1 plays an important role in C/EBP β -2-mediated transactivation of the subtype C LTR. It remains possible, however, that the extra NF- κ B binding site or some other transcription factor binding sites within the subtype C LTR impacts C/EBP-mediated function and plays a role in compensating for C/EBP binding site differences between subtype B and C LTRs. For example, the transcriptional activation of the HIV-1 LTR by p50-C/EBP β heterodimeric complexes has been demonstrated within subtype B [26] and this functional interaction has been shown to be dependent on the DNA-binding ability of p50 and the transcription activation domain of C/EBP β . With regard to the subtype C LTR, three NF- κ B binding sites may result in enhanced binding of C/EBP β -NF- κ B heterodimeric complexes to the LTR and result in increased activation of the LTR. Whether the C/EBP sites are required for HIV-1 subtype C replication in monocyte-macrophage cell lineages

remains to be elucidated. The level of basal transcription driven by the representative subtype C LTR was higher than that of the representative subtype B LTR in U-937 monocytic cells (Fig. 4), which is consistent with previous studies that have been performed in Jurkat T-cell lines [13], suggesting that the role of the extra NF- κ B site in subtype C LTR basal activity is independent of cell type.

Different HIV subtypes have been shown to exhibit different biologic properties, pathogenesis, disease progression, and antiretroviral drug resistance [60–64]. Specifically, differences in selected LTR regions may be associated with alterations in virus transcription, replication, and disease progression [6,10]. Interestingly, the subtype B Sp III-5T sequence variant (C-to-T change at position 5 compared with the consensus B Sp site III) that has been shown to be correlated with HIV-1 subtype B disease progression [65] appeared in the subtype C Sp site III consensus sequence. Whether this LTR sequence alteration is related to HIV-1 subtype C disease progression will require additional clinical information, viral genotyping, and molecular pathogenesis analyses to definitively delineate this potential structure/function correlation. The conservation of subtype C Sp site I was relatively low (36%) in the analysis, which is different from other reports showing that subtype C Sp site I [8] and Sp site II are the most conserved [66] among the three Sp sites. The number of LTRs examined in these studies was less than the number used in the present studies, however, and the function of Sp sites or the GC box array in subtype C gene expression needs additional investigation. Unexpectedly, subtype C virus exhibited a relatively high degree of conservation in the ATF/CREB site (percentage of consensus sequence: 93.9%, Fig. 3), which was comparable to that of the NF- κ B sites. The function of the ATF/CREB site in subtype C gene expression is not known. Subtype B ATF/CREB site variants (TGACACAA, including C-to-A and G-to-A changes at position 7 and 8, respectively), however, were able to affect subtype B LTR basal activity and LTR function following interleukin-6 stimulation, a treatment that leads to increases in C/EBP β activation [30]. This variant sequence is also similar to the subtype C ATF/CREB consensus sequence (TGACACAG). Therefore, it is possible that the subtype C ATF/CREB site is able to affect LTR basal transcription or transcription mediated by its corresponding transcription factors or the nearby NF- κ B sites. Phenotypically, selected consensus sequences of subtype C (Sp site III 5T, ATF/CREB site 7C and 8C) have been found in subtype B as naturally occurring sequence variations and have been shown to exhibit low DNA binding ability. Others have reported that sequence variations in one transcriptional binding site affected DNA binding affinities of other neighboring transcriptional binding sites and viral proteins, and ultimately the promoter activation potential of subtype B LTRs [30,31]. Thus, it is possible that the replacement of C/EBP US1 and the low DNA binding affinity of the ATF/CREB and Sp site III binding sites within the subtype C LTR act together to decrease DNA binding competition with NF- κ B, resulting in a better structural environment for NF- κ B factors to bind the enhancer element rapidly and efficiently, which might be one of the factors contributing to subtype C replication and pathogenesis. Perhaps these specific LTR alterations facilitate viral replication in cells of the monocyte-macrophage lineage and enhance viral transmission capabilities.

As reported in other studies, subtype C LTR activities are enhanced in the presence of subtype C Tat in HEK293 cells and in Jurkat T cells [13,50,67]. The studies reported here have shown that subtype C LTR activities are also activated by subtype C Tat in U-937 monocytic cells (Fig. 5). The physical and functional interaction between C/EBP β and subtype B Tat-regulating gene expression has been reported [27,32,33,68]. The cooperation of subtype B Tat and C/EBP β has been shown to increase C/EBP β DNA binding affinity for the HIV-1 LTR [27,33] and to regulate transcription of monocyte chemoattractant protein 1 in astrocytes [32]. Consequently, it has been proposed that subtype B Tat and C/EBP β interaction contributes to HIV-1-associated brain dysfunction in patients with HIV-1

subtype B. Studies to date have shown, however, that HIV-1 subtype C is associated with a lower prevalence of HIV-1-associated dementia in India [51], although the exact role of subtype C in neurologic disease requires further investigation. Our results have demonstrated that subtype C Tat maintains transactivation capability and the ability to interact with other transcription factors, such as C/EBP β , in U-937 cells. Thus, the interaction of subtype C Tat and C/EBP may not be associated with low occurrence of HIV-associated dementia. As reported previously [51,52,67], the relatively low occurrence of HIV-associated dementia in individuals infected with subtype C virus might be caused by other properties of subtype C Tat, including deficient chemoattractant properties, low binding affinity for chemokine receptor 2 (CCR2), or an inability to stimulate tumor necrosis factor- α production as a result of a serine-to-cysteine substitution at amino acid position 31.

In summary, two C/EBP binding sites have been identified within the HIV-1 subtype C LTR. Additional studies have suggested that transactivation ability of C/EBP β may play an important functional role in the pathogenesis of different HIV-1 subtypes. Our results also suggest that viral LTR sequence variation in different subtypes as a result of viral genomic alterations and host selective pressures may be associated with differences in viral replication and disease progression. Further studies will determine how C/EBP US1 and DS1 affect subtype C gene expression in cells of monocyte-macrophage lineage and examine the roles of conserved ATF/CREB and Sp binding sites in HIV-1 subtype C gene expression.

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Fig. 1.

Members of the C/EBP transcription factor family bind to subtype C LTR. (A) The location of three putative C/EBP binding sites in subtype C LTR and four C/EBP binding sites within subtype B LTR are shown. (B) Sequence comparison of C/EBP sites between subtype B and C. (C) Consensus subtype C-US1 (ConC-US1) was able to form DNA-protein complexes containing C/EBP α , β , and δ . Supershift EMS analyses were performed as described in "Materials and Methods." Radiolabeled ConC-US2, ConC-US1, and ConC-DS1 were used as probes and reacted with U-937 nuclear extract in the absence or presence of antibodies specific to C/EBP α , β , γ , ϵ , and δ . Arrows show the specific DNA-protein complexes.

LAI AAGCTAGTACCAGTTGAGCCAGATAAGGTAGAAGAGGCCAATAAAGGAGAGAACACCAGCTTGTACACC 210
ConC AAGCTAGTGCCAGTTGACCAAGGGAAGTAGAAGAGGCCAACGAAGGAGAAAACAACCTGTTGTACACC 210

US3

LAI CTGTGAGCCTGCATGGAATGGATGACCTGAGAGAGAAGTGTAGAGTGGAGTTTGACAGCCGCCTAGC 280
ConC CTATGAGCCAGCATGGAATGGAGATGAAGACAGAGAAGTATTAAGTGGAAAGTTTGACAGTCACCTAGC 280

US1

LAI ATTTTCATCACGTGGCCGAGAGCTGCATCCGGAGTACTTCAAGAAC--TGCTGACATCGAG---CTTGC 344
ConC ACGCAGACACATGGCCCGAGCTACATCCGGAGTATTACAAGACACTGCTGACACAGAAGGACTTTC 350

US2 **US1**

LAI TACAAGGGACTTTCCGCTGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCC 414
ConC CGCTGGGACTTTCCACTGGGG-CGTTCCAGG-AGGTGTGGTCTGGGCGGGACTGGGGAGTGGTCAACC 417

ATF/CREB **NF-κB III**

LAI TCAGATGCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTGGTTAGACCAGATTGAGCCT 484
ConC TCAGATGCTGCATATAAGCAGCTGCTTTTGCCTGTACTGGGTCTCTCTAGGTAGACCAGATCTGAGCCT 487

NF-κB II **NF-κB I** **Sp III** **Sp II** **Sp I**

LAI GGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTGAGTGCTTCAAGT 554
ConC GGGAGCTCTCTGGCTATCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCCTTGAGTGCTTCAAGT 558

LAI AGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACCCCTTTAGTCAGTGTGGAAA 624
ConC AGTGTGTGCCCGTCTGTTGTGTGACTCTGGTAACTAGAGATCCCTCAGACCCCTTTNNGGTAGTGTGGAAA 628

DS1

LAI ATCTCTAGCA 634
ConC ATCTCTAG 636

Fig. 2.

Alignment and comparison of consensus sequences of indicated transcription factor binding sites within the LTR of HIV-1 subtypes B and C. Representative transcription factor binding sites within the HIV-1 subtype B and subtype C consensus sequences are shown. Arrow indicates the transcriptional start site.

Fig. 3.

Prevalence of the consensus configurations of indicated transcription binding sites between subtypes B and C. (A) Selected transcription binding sites within HIV-1 consensus subtype B (ConB) and C (ConC) LTRs. The arrow indicates the transcriptional start site. (B) HIV-1 subtype B LTRs from three published papers [55–57] and subtype C LTRs from GenBank and Los Alamos databases were compiled and aligned using the Clustal W method. The number shown indicates the percentage of each of the indicated transcription binding sites with the respective consensus configuration present in the total HIV-1 LTR sequences available for inspection.

Fig. 4. C/EBPβ-2 is able to activate the HIV-1 subtype C LTR. (A) and (C) U-937 monocytic cells were transiently transfected with luciferase reporter constructs under the control of subtype B or subtype C parental LTR in the absence (A) or presence of C/EBPβ-2 expression vector (C). Relative luciferase activity was quantitated 24 h after transfection. Results are shown as “fold” difference over the parental subtype C LTR. (B) C/EBPβ-2 isoform structure. Asterisk indicates the significant difference (p value <0.001). Error bars indicate the standard deviation for each data point.

Fig. 5.

The activities of HIV-1 subtype C LTR caused by Tat and C/EBPβ-2 in U-937 cells. U-937 cells were transiently transfected with parental subtype C LTR in the absence or presence of C/EBPβ-2, Tat alone, or in combination. Twenty-four hours after transfection, cell lysates were collected and followed by protein quantification and luciferase activity assays. Results are shown as “fold” difference over parental LTR. Error bars indicate the standard deviation for each data point.