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Identification of Novel T Cell Factor 4 (TCF-4) Binding Sites on the HIV Long Terminal Repeat Which Associate with TCF-4, β-Catenin, and SMAR1 To Repress HIV Transcription

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Molecular regulation of HIV transcription is a multifaceted process dictated in part by the abundance of cellular transcription factors that induce or repress HIV promoter activity. β-Catenin partners with members of the T cell factor (TCF)/LEF transcription factors to regulate gene expression. The interaction between β-catenin and TCF-4 is linked to inhibition of HIV replication in multiple cell types, including lymphocytes and astrocytes. Here, we evaluated the molecular mechanism by which β-catenin/TCF-4 repress HIV replication. We identified for the first time multiple TCF-4 binding sites at −336, −143, +66, and +186 relative to the transcription initiation site on the HIV long terminal repeat (LTR). Two of the sites (−143 and +66) were present in approximately 1/3 of 500 HIV-1 isolates examined. Although all four sites could bind to TCF-4, the strongest association occurred at −143. Deletion and/or mutation of −143, in conjunction with β-catenin or TCF-4 knockdown in cells stably expressing an LTR reporter construct, enhanced basal HIV promoter activity by 5-fold but had no effect on Tat-mediated transactivation of the HIV LTR. We also found that TCF-4, β-catenin, and the nuclear matrix binding protein SMAR1 tether at the −143-nucleotide (nt) site on the HIV LTR to inhibit HIV promoter activity. Collectively, these data indicate that TCF-4 and β-catenin at −143 associate with SMAR1, which likely pulls the HIV DNA segment into the nuclear matrix and away from transcriptional machinery, leading to repression of basal HIV LTR transcription. These studies point to novel avenues for regulation of HIV replication by manipulation of β-catenin signaling within cells.

Transcriptional activity of human immunodeficiency virus type 1 (HIV-1) depends on both *cis* and *trans* elements that regulate the 5′ long terminal repeat (LTR) of the HIV promoter. The promoter is divided into four regions: the TAR element, the core promoter, the core enhancer, and the modulatory region, which contains a negative regulatory element (NRE) (25). A number of transcription factors induce HIV promoter activity, such as C/EBP, NF-κB, NFAT, AP-1, and Sp1 (7, 13, 19). Others are suppressors of HIV promoter activity, the most notable of which are YY-1, LSF, p50 homodimer, CBF-1, CTIP2, and c-myc (5, 9, 18, 23, 35, 37). These transcriptional factors negatively regulate the HIV LTR by recruiting histone deacetylases (HDACs) and methyltransferases to alter the chromatin structure to render it inaccessible to the transcriptional machinery and hence play a significant role in repression of HIV transcription. We and others have identified an additional transcriptional factor (T cell factor 4 [TCF-4]) that inhibits HIV replication (3, 26, 28, 38). The mechanism by which TCF-4 mediates HIV repression is not entirely clear and is the focus of this study.

TCF-4 is a downstream effector of the canonical Wnt/β-catenin pathway. It associates with β-catenin (a transcriptional coactivator) to regulate the transcription of a diverse set of genes involved in cell proliferation, differentiation, communication, and survival. The Wnt/β-catenin pathway is initiated by binding of Wnt ligands (small secreted glycoproteins) to members of the Frizzled family of seven-transmembrane G protein-coupled receptors and a coreceptor, such as low-density lipoprotein receptor-related protein 5 or 6 (LRP 5/6). Receptor/ligand binding initiates a complex signal transduction cascade that results in destabilization of a multiprotein β-catenin destruction complex. The net result is the accumulation of a stable, hypophosphorylated form of β-catenin that can translocate to the nucleus. Within the nucleus, β-catenin displaces negative regulatory elements from TCF-4, such as transducin-like enhancer protein 1 (TLE1) and HDACs, and recruits coactivators, such as BCL9, Pygopus, and CBP/p300, to activate transcription of Wnt target genes. In the absence of Wnt ligands, β-catenin is constitutively phosphorylated and targeted for degradation by the destruction complex (1, 14). Although β-catenin/TCF-4 association is classically linked to target gene activation, recent evidence indicates that under certain circumstances, β-catenin/TCF-4 association functions as a transcriptional repressor (16). Indeed, we previously described a role for β-catenin/TCF-4 in repression of HIV replication in multiple cell types, including peripheral blood lymphocytes and astrocytes (3, 20, 22, 26). Astrocytes have abundant β-catenin/TCF-4 signaling, which makes them an ideal system to evaluate the mechanism by which β-catenin/TCF-4 suppress HIV replication and to probe the role of β-catenin/TCF-4 signaling in HIV-mediated pathogenesis in the central nervous system (CNS).

Astrocytes are latently infected by HIV (6, 11, 27, 33, 34, 36). Typically, astrocytes support a low level of productive HIV repli-

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cation in comparison to other CNS cellular targets of HIV, such as microglia and perivascular macrophages. In response to signals that inhibit the Wnt/β-catenin pathway, astrocytes support a much higher level of HIV replication. For example, gamma interferon (IFN-γ), a cytokine that is elevated in the cerebrospinal fluid (CSF) of patients with HIV-associated dementia (HAD) and is released by activated microglia, macrophages, infiltrating lymphocytes, and astrocytes (21), induces a 4-fold increase in HIV replication by inhibiting β-catenin/TCF-4 signaling through induction of an antagonist of the β-catenin pathway (DKK1) in a Stat3-dependent manner (2, 3, 22). Because the degree of astrocyte infection correlates with the severity of HIV-mediated neuropathogenesis and proximity to microglia/macrophages (4), it is likely that signals mediated by microglia/macrophages (e.g., IFN-γ or others that perturb β-catenin/TCF-4 signaling) may regulate permissiveness to HIV productive replication in astrocytes.

Astrocytes perform vital functions to maintain brain homeostasis, from metabolic breakdown of potentially excitotoxic transmitters to maintaining blood brain barrier integrity. Their dysregulation is one of the hallmarks of the severity of HAD (24). However, our knowledge of HIV regulation at the transcriptional level in these cells is incomplete. In particular, little is known about the role of β-catenin/TCF-4 in HIV transcriptional activity in astrocytes. Here, we evaluate the mechanism by which TCF-4 mediates inhibition of HIV transcription in astrocytes. We demonstrate for the first time that the HIV LTR promoter has functional binding sites for TCF-4, which associates with β-catenin and the nuclear matrix protein SMAR1 to create a repressive complex for HIV transcription. While these findings are explored in astrocytes, the phenomenon observed is relevant in a number of model systems where β-catenin signaling is intact. Our studies also highlight a role for exploring the contribution of β-catenin/TCF-4 to HIV latency and as a novel pathway for anti-HIV therapy.

MATERIALS AND METHODS

Cell culture. The U87MG astrocytoma cell line was obtained from the NIH AIDS Research and Reference Reagent Program (Frederick, MD). The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Sigma, St. Louis, MO) and 1% penicillin-streptomycin (Gibco Invitrogen) in a 5% CO_2 humidified atmosphere at 37°C. Adherent cells were removed by treatment with 1 mM EDTA for 5 min with gentle scraping or pipetting multiple times.

Stable integration of LTR-Luc in cell lines. Stable cell lines expressing LTR-driven luciferase (LTR-Luc) were generated as previously described (26). Briefly, U87MG cells were transfected with a wild-type (WT) LTR plasmid derived from HIV-1_{Bal} sequence. The plasmid is from a pGL4.19 background with the neomycin gene as a marker gene. After 48 to 72 h of transfection, the cells were collected, washed with phosphate-buffered saline (PBS), and cultured in the presence of G418 (25 μg/ml) at a low cell density (20 to 40% confluence) until they reached full confluence, which was typically within 4 to 6 days. G418 was added at day 2 after seeding. Subsequently (4 to 6 days after seeding), the cells were detached. These were considered passage 1 cells. The cells were then replated as indicated above in the presence of G418 treatment, and this process was repeated for several generations until the episomal plasmid was diluted out and only the chromosomally integrated HIV LTR reporter that can drive neomycin resistance remained. The presence of HIV LTR and neomycin genes was confirmed by PCR, while luciferase was confirmed by the luciferase reporter assay.

Bioinformatics search for TCF-4 DNA binding sequences in HIV LTR. HIV_{Bal} was sequenced with primers flanking LTR (the R and U5 regions), TAR, and the start of the *gag* gene. Primer design was based on clone pBa-L (accession number AB221005). The AliBaba2 program and TRANSFAC database were used to predict binding sites for transcription factors in the HIV LTR promoter. Additionally, for searching putative binding sites containing single-base substitutions, we employed the alignment tool in Vector NTI and OMIGA. The TCF-4 consensus sequence should include a 5′-(A/T)(A/T)CAAAG-3′ stretch (32, 39).

EMSA and supershift assay. Double-stranded DNA probes with or without biotin conjugation at the 5′ end were purchased from Integrated DNA Technologies (IDT) (San Diego, CA). The sequences of the probes were Bal−336, 5′-CACTGACCTTTGGATGGTGCT; Bal−143, 5′-GGAGTACTACAAAGACTGCT; Bal+66, 5′-AGCCTCAATAAAGCTTGCCT; Bal+186, 5′-CCCGAACAGGGACTTGAAAGCGAAAGAGAA; and Bal−143 scrambled, 5′-GGAACAAGTTCCGGCACGCT. Nuclear extract (NE) was prepared from U87MG cells, according to instructions provided in the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). The protein content was determined with a Pierce BCA protein assay kit. For DNA-protein binding, 10 μg of NE and 100 ng of probe were incubated for 15 min at room temperature in a total volume of 10 μl of binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 4% glycerol). The DNA-protein complexes were run on a 6% native gel and detected with SYBR green dye using an electrophoretic mobility shift assay (EMSA) kit, as recommended by the manufacturer (Invitrogen). For supershift assays, the NE was first incubated with 1 μg of TCF-4 antibody or a suitable IgG control for 15 min, and then 100 ng of DNA probe was added.

Biotin-streptavidin DNA-protein pulldown assay. A DNA-protein affinity assay was performed under low- and high-salt washes. NEs at 100 μg were incubated with 200 ng of biotinylated DNA probes (corresponding to TCF-4 binding sequences identified at −336, −143, +66, or +186 relative to the transcription initiation site in HIV_{Bal}) for 5 min at room temperature with mild mixing. Streptavidin-conjugated agarose beads were added, and incubation was continued for another 30 min with slow mixing. The beads were then pelleted, washed three times with TNE buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 100 to 300 mM NaCl), and suspended in SDS loading buffer, and equal volumes were run on a 10% SDS electrophoresis gel. The proteins were then transferred onto a nitrocellulose membrane and immunoblotted with a TCF-4 antibody (mouse monoclonal antibody [MAb]; Cell Signaling, Boston, MA).

Plasmid constructs and site-directed mutagenesis. Genomic DNA was obtained from HIV-1_{Bal}-infected peripheral blood mononuclear cells (PBMCs) (after 3 days of infection) using a DNeasy blood and tissue kit (Qiagen, Germantown, MD). The LTR was amplified using the primers Sn25 (5′-TCGACTCGAGGACAAGATATCCTTGATTTGT) and Sn26 (5′-TCGACTCGAGTTTGGCGTACTCACCAGTCG) cut with XhoI (underlined sequence) and cloned into XhoI-predigested pGL4.19 plasmid (Promega, Madison, WI). The recombinant plasmid was subjected to digestion with endonucleases SfiI and EcoRI to confirm the presence of a single copy of the LTR insert and further sequenced to confirm its proper orientation and was termed WT-LTR. WT-LTR was subjected to site-directed mutagenesis using the QuikChange multikit (Stratagene) to obtain the Δ-143-LTR (primers Sn30, 5′-GCTGTATCCGGAGTACCTGCTGACATTGAGCT, and Sn31, 5′-AGCTCAATGTCAGCAGGTACTCCGGATACAGC) and mut-143-LTR (primers Sn32, 5′-GTGGCCCGAGAGCTGTATCCGGAGTACGCTGCGAACTGCTGACATTGAGCTTTCTACAAGG, and Sn33, 5′-CTTGTAGAAAGCTCAATGTCAGCAGTTCGCAGCGTACTCCGGATACAGCTCTCGGGCCA) plasmids. All plasmids were sequenced individually from both directions to confirm proper deletions/mutations.

Transfections and reporter assays. Transfections of generated plasmids with or without the indicated small interfering RNAs (siRNAs) were performed using TransIT-LT1 reagent according to the manufacturer's instructions (Mirus Bio LLC, Madison, WI). On-Target*plus* Smartpool siRNAs specific for TCF-4, β-catenin, SMAR1/BANP, and scrambled siRNA were obtained from Thermo Scientific and transfected into cells using Lipofectamine siRNAmax (Invitrogen) according to the reagent

protocol. The cells were approximately 60 to 70% confluent at the time of transfection. Approximately 24 h after transfection, construct reporter activity was performed using a dual-luciferase reporter assay (Promega, Madison, WI). Briefly, the medium was removed, the cells were gently washed with PBS, 100 μl of passive lysis buffer was added, the cells were incubated at 37°C for 10 to 12 min and centrifuged at 5,000 rpm for 4 min to remove debris, and 10 to 20 μl of lysate was used to assay for firefly and *Renilla* luciferase activity in a single-injector luminometer. Relative light units were normalized to a *Renilla* luciferase control or μg/ml of protein as indicated. The total protein concentration was measured using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific).

Protein immunoprecipitation and Western blotting. Nuclear lysates from U87MG cells were prepared using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). Lysates were first precleared by incubation with magnetic A/G beads (Thermo Scientific) and a nontargeting rabbit IgG (Cell Signaling) for 1 h. The lysates were then incubated with magnetic A/G beads and with either IgG control, TCF-4 antibody (Cell Signaling), SMAR1 antibody (Abcam), or β-catenin antibody (Sigma) overnight at 4°C with rotation. The beads were washed extensively with Tris-buffered saline (TBS) supplemented with 2 M urea, followed by elution with low-pH buffer (pH 2.9; GE Healthcare). Additionally, to minimize nonspecific interference from detection of antibody heavy chain in Western blotting, TCF-4 antibodies and their cognate control antibodies were cross-linked to magnetic A/G beads by incubation with 200 mM triethanolamine (GE Healthcare) containing 50 mM dimethyl pimelimidate dihydrochloride (DMP) (Sigma) prior to immunoprecipitation. For Western blotting, immunoprecipitated samples were separated on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with Superblock (Thermo Scientific) containing 0.1% Tween 20 (T20) for 1 h, and incubated with the indicated antibody overnight at 4°C (SMAR1 and β-catenin, 1:5,000; TCF-4, 1:1,000) in Superblock-0.1% T20. The membranes were washed extensively with Tris-buffered saline with Tween 20 (TBST) and incubated with secondary antibody conjugated to horseradish peroxidase (HRP) (1:50,000 in Superblock-0.1% Tween 20) for 45 min at room temperature (RT). The membranes were again washed extensively in TBST and developed with SuperSignal West Femto maximum-sensitivity substrate according to the manufacturer's instructions (Thermo Scientific). To test for the specificity of SMAR1 antibody binding, SMAR1 antibody was incubated with a 5-fold excess of SMAR1/BANP blocking peptide (Abcam) for 30 min prior to Western blotting.

ChIP and quantitative real-time PCR. U87MG cells were transiently transfected with WT-LTR or Δ-143-LTR plasmid, and after 24 h, chromatin immunoprecipitation (ChIP) was performed on the cells using a Millipore kit with antibodies for TCF-4 (Cell Signaling), LEF1 (Cell Signaling), β-catenin (Sigma), or SMAR1/BANP (Abcam). A suitable IgG (Cell Signaling) control antibody was also included in the experiments. The primers used to amplify LTR were Sn57 (5′-TGGAAGTTTGACAGC ACCCTAGCA) and Sn58 (5′-AGCATCTGAGGGCTCGCCA) to amplify a 165-bp region encompassing nt −143, as well as Sn59 (5′-AAGTAGTG TGTGCCCGTCTGTTGT) and Sn60 (5′-AGAGCTCCTCTGGTTTCTC TTTCGCT) to amplify a 133-bp region encompassing nt +186. As a negative control, untransfected U87MG cells that did not contain HIV DNA were subjected to ChIP analysis to ensure that the chosen primers did not amplify genomic DNA. Real-time PCR was performed using Ssofast Evagreen Supermix with a Low Rox kit (Bio-Rad, Hercules, CA) in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using 7500 software v2.0.1. Melting curve analysis was performed to ensure the amplification of a single product. The change in binding was calculated by relative quantification using the comparative threshold cycle (C_T) method, with results reported as fold change relative to control ($\Delta C_T = C_{T\text{Target}} - C_{T\text{IgG control}}$; fold change relative to IgG control $= 2^{-\Delta C_t}$).

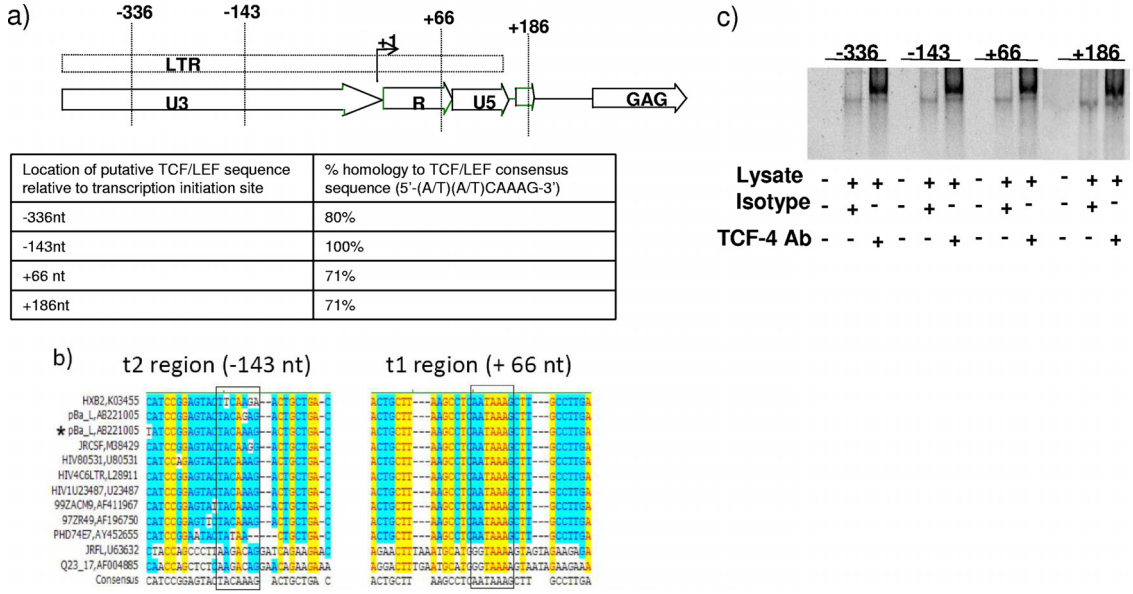
Nucleotide sequence accession number. The sequence of the LTR was submitted to GenBank (accession number HQ882192).

RESULTS

Identification of several TCF-4 binding sites in the HIV LTR. We searched for putative binding sites within the HIV_{Bal} LTR for the core consensus sequence of TCF-4 binding (5′-[A/T][A/T]CAAAG-3′) (32, 39). Depending on the gene, this core binding sequence can be found in both direct and reverse orientations within the promoter. This strategy identified 4 putative TCF-4 binding sites (Fig. 1a). The locations of these sites from the transcription initiation site are as follows: 1, nt −143 to −136; 2, nt −336 to −329; 3, nt +66 to +73; and 4, nt +186 to +195. The site at position +186 is within the *gag* leader sequence and is just outside the promoter. The site at nt −143 has 100% homology to the TCF-4 consensus sequence, the site at nt −336 has 80% homology, and the sites at nt +66 and +186 have 71% homology to the TCF-4 binding sequence (Fig. 1a). To address the level of polymorphism in TCF-4 putative binding sites among different HIV-1 strains, we aligned HXB2, pBaL, and other HIV-1 proviral DNA with the sequence of the HIV-1 LTR. We found that two of the four TCF-4 binding sequences identified (−143 and +186) were present in approximately one-third of 500 HIV LTR sequences reported in the Los Alamos gene bank. A representative sample of TCF/LEF binding sites found in several HIV isolates is depicted in Fig. 1b. EMSAs and supershift assays confirmed that all four putative TCF-4 sites can bind to TCF-4 protein (Fig. 1c).

Higher affinity of TCF-4 binding to nt −143 in the HIV LTR promoter. To assess whether a particular site has a higher affinity for TCF-4 binding, we utilized a biotin-streptavidin-based pull-down assay, followed by a stringent wash with NaCl (100 to 300 mM) (8). Washing with 100 mM NaCl did not show differential binding among all four sites. However, washing with 300 mM NaCl showed 2-fold- and 4-fold-stronger binding affinities for the TCF4 78-kDa and 59-kDa isoforms, respectively, at nt −143 (Fig. 2a and b). These studies demonstrated that both isoforms of TCF-4 have stronger binding affinity at nt −143 than at nt −336, +66, and +186. These data are consistent with the observation that site −143 has 100% homology to the consensus TCF-4 DNA binding sequence (Fig. 1a). Further, TCF-4 bound to sites −336, +66, and +186 with approximately equivalent affinities, which were lower than observed at −143 but were still readily detected (Fig. 2b). We chose to focus our efforts on the TCF-4 binding sites at −143 and +186 in the HIV LTR due to their presence in a wide variety of isolates, their proximity to regions known to impact HIV transcription, and the strength of TCF-4 binding to the −143 site.

Tethering of β-catenin and TCF-4 at nt −143 on the HIV LTR. To assess tethering of TCF-4 on the HIV LTR at nt −143 and +186 *in vivo*, we transfected U87MG astrocytoma cells with HIV_{Bal} LTR plasmid linked to a luciferase reporter. This wild-type HIV LTR_{Bal} plasmid contains all four TCF-4 consensus sequences, including nt −143 and nt +186. We then performed ChIP assays using anti-TCF-4 antibody or isotype control and amplified for HIV sequences at nt −143 or +186. We demonstrate that TCF-4 is tethered at nt −143 at a higher level than in the nt +186 region (Fig. 3a). Because TCF-4 and LEF1 can compete for the same binding sites and because, unlike TCF-4, LEF1 is an inducer of HIV LTR activity (30), we evaluated the tethering of LEF1 at nt −143 and nt +186 in comparison to TCF-4 binding at these sites. LEF1 binding was detectable at very low levels (1.5-fold over the IgG control) at both −143 and +186 (Fig. 3a). Although statistically significant, this level of binding is unlikely to be highly rele-



Location of putative TCF/LEF sequence relative to transcription initiation site	% homology to TCF/LEF consensus sequence (5'-(A/T)(A/T)CAAAG-3')
-336nt	80%
-143nt	100%
+66 nt	71%
+186nt	71%

FIG 1 TCF-4 binds to its putative binding sites on the HIV LTR *in vitro*. (a) HIV_{Bal} was sequenced using primers that spanned from the LTR into the start of the *gag* gene. AliBaba2 software was used to predict putative TCF-4 binding sites on the HIV LTR using the consensus sequence 5′-(A/T)(A/T)CAAAG-3′. Alignment tools in Vector NTI and OMIGA software were used to find putative binding sites containing single-base substitutions. (b) Multiple-LTR-sequence alignment to scan for putative TCF-4 binding sites in 500 HIV-1 isolates from the Los Alamos gene bank. A representative sample shows the presence of two TCF-4 sites at nt −143 and +66 in several isolates. The yellow highlights TCF-4 bases that are highly conserved (unchanged), while less-conserved bases are in turquoise. The boxes show TCF-4 positions at −143 and +66. The asterisk denotes sequenced HIV_{Bal} from panel a that contains point mutations not found in the Bal isolate from the database (pBa_L; AB221005). (c) EMSA indicating binding of TCF-4 at these four sites. Nuclear extract (lysate) from U87MG cells was incubated with the indicated biotinylated DNA probe for 15 min. DNA-protein complexes were run on a 6% native gel and detected with SYBR green dye. For supershift assays, nuclear extract was incubated with IgG control (isotype) or TCF-4 antibody for 15 min prior to incubation with DNA probes. The data in panel c are representative of three independent experiments.

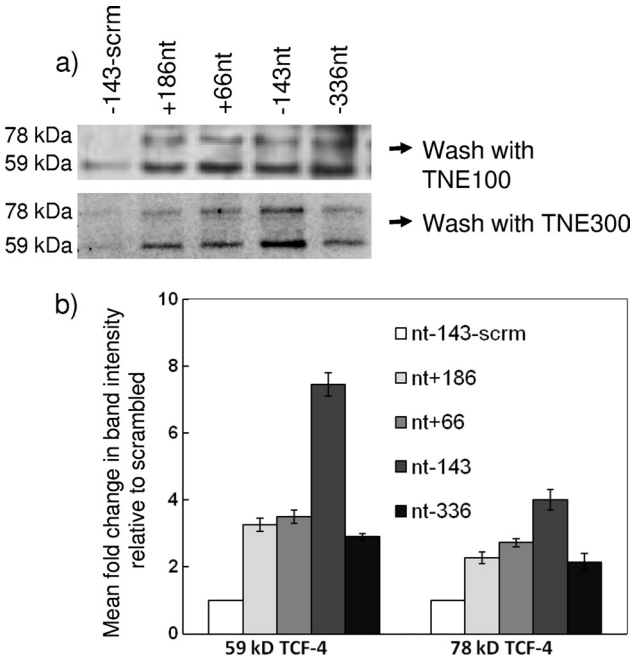


FIG 2 TCF-4 exhibits stronger binding at the −143 site on the HIV LTR. (a) Nuclear extract from U87MG cells was mixed with the indicated biotinylated DNA probe for 5 min, followed by incubation with streptavidin-conjugated agarose beads. The beads were pelleted and washed three times with TNE100 or TNE300. Samples were then run on an SDS-PAGE gel and immunoblotted to detect TCF-4. (b) Densitometric analysis of bands detected in panel a showing relative binding of TCF-4 following washing with TNE300. The data are normalized to scrambled control and represent three independent experiments. The error bars represent standard deviations (SD).

vant to transcription and can be considered negligible. Deletion of the nt −143 site largely abolished TCF-4 binding to the LTR (Fig. 3b). These studies demonstrate that TCF-4 specifically and preferentially binds to the nt −143 site on the HIV LTR.

Given recent evidence that β-catenin/TCF-4 binding can repress rather than induce gene expression (16), we evaluated the role of β-catenin in association with TCF-4 in repression of HIV transcription. We demonstrate by ChIP that β-catenin is also tethered on the HIV LTR at nt −143 (4.1-fold over control IgG), with less binding observed at nt +186 (2.7-fold over control) (Fig. 3a). As expected, β-catenin was not detected on HIV LTR flanking the −143 region when nt −143 was deleted (Fig. 3b). To ensure that no amplicons were detected in the absence of HIV DNA, we also performed ChIP on untransfected cells. We did not detect any HIV LTR amplicons in cells lacking the HIV LTR construct (data not shown). Collectively, these data indicate that β-catenin and TCF-4 preferentially bind the HIV LTR at nt −143 *in vivo*.

Impact of mutations and/or deletions of the nt −143 TCF-4 binding site on basal HIV LTR promoter activity. Given our identification of TCF-4 binding sites on the HIV LTR and tethering of TCF-4 at nt −143, we evaluated the impact of deletion and/or mutation of these two sites on the HIV promoter in transient-transfection assays using the wild-type LTR reporter construct or a construct with position nt −143 deleted (26) (Fig. 4a). Although statistically significant, deletion or mutation of nt −143 only modestly reduced HIV promoter activity in transient transfections (data not shown).

HIV transcription is likely to be regulated by epigenetic modifications that are underappreciated in the context of transient-

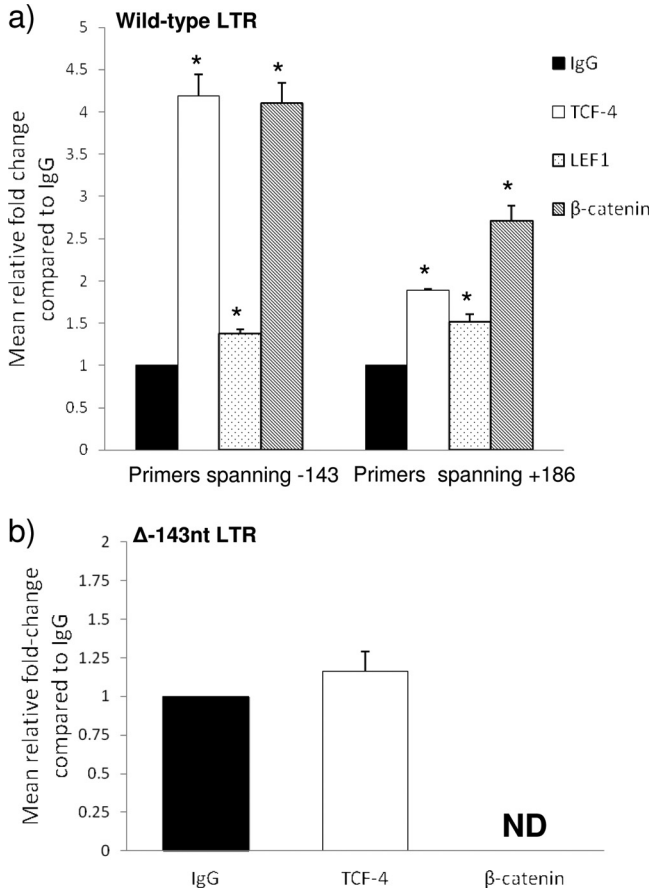


FIG 3 TCF-4 docks on HIV LTR *in vivo* and exhibits stronger binding affinity at nt −143. (a) U87MG cells were transfected with a wild-type LTR construct. Twenty-four hours later, ChIP was performed using the indicated immunoprecipitating antibodies. Eluted DNA was analyzed by real-time PCR using primers encompassing nt −143 (Sn57 and Sn58) and nt +186 (Sn59 and Sn60) for quantitative amplification. (b) U87MG cells were transfected with an HIV LTR with the nt −143 TCF-4 binding site deleted (Δ-143-LTR). Twenty-four hours later, chromatin immunoprecipitation was performed, followed by real-time PCR, as shown in panel a. All data are expressed as mean fold change relative to a control IgG antibody, as described in Materials and Methods. The data represent at least three independent experiments. *, $P < 0.05$ compared to IgG control; ND, not detected. The error bars represent SD.

transfection assays. To determine whether repression of HIV transcription by β-catenin/TCF-4 involves modification of a larger chromatin structure, we generated stably transfected cell lines with wild-type LTR or LTR with nt −143 deleted with or without siRNA for β-catenin or TCF-4 (26). The level of β-catenin or TCF-4 knockdown was consistently greater than 90% (26). While knockdown of β-catenin or TCF-4 modestly enhanced LTR activity in transient-transfection assays (1.8 to 2.8-fold) regardless of whether the nt −143 site was present (Fig. 4b), stable expression of the Δ-143-LTR construct significantly increased the ability of a β-catenin or TCF-4 knockdown to induce HIV transcription (4.8- to 5-fold induction in siRNA-transfected Δ-143-LTR cells versus 2-fold induction in WT LTR cells) (Fig. 4c). Similar results were obtained with an LTR-luciferase construct containing a scrambled TCF-4 binding site at −143 (data not shown). Knocking down both β-catenin and TCF4 did not further enhance LTR activity (Fig. 4d), suggesting that these factors work together to repress HIV transcription. These data highlight the relevance of the nt −143 position in suppressing HIV basal transcriptional activity in association with β-catenin and TCF-4 and suggest that chromatin remodeling is involved in the mechanism of repression.

Impact of the nt −143 position on Tat transactivation of HIV LTR. Basal HIV transcriptional activity is inefficient due to the recruitment of a poorly processive RNA polymerase II (Pol II) to the HIV LTR, resulting in accumulation of short, abortive transcripts. In order for efficient replication to occur, the viral regulatory protein Tat must recruit a positive elongation complex to Pol II to enhance its processivity. We evaluated the impact of the nt −143 site on Tat transactivation of the HIV LTR, with or without knockdown of β-catenin or TCF-4, using LTR-luciferase reporters. While, as expected, transfection with Tat alone increased WT and Δ-143-LTR activity under both the episomal and integrated conditions, the level of induction was much greater with transient transfection (Fig. 5a and b). The level of induction was further enhanced by knockdown of either TCF-4 or β-catenin (Fig. 5a and b). In particular, knockdown of β-catenin or TCF-4 had a profound effect on HIV promoter activity when the LTR was integrated into the genome (3.8- to 6.6-fold induction relative to Tat alone) versus transiently transfected LTR (1.4- to 3.1-fold) (Fig. 5a and b), suggesting that chromatin remodeling is involved in the ability of β-catenin/TCF-4 to regulate Tat-mediated transactivation. However, deletion of the TCF-4 binding site at −143 had no significant impact on Tat-induced LTR activity, suggesting that the site is not involved in regulating Tat-mediated transactivation of the HIV promoter but is involved in regulating basal HIV LTR transcription.

Role of SMAR1 in suppression of HIV LTR activity at site nt −143. The nuclear matrix functions as a scaffold to organize chromatin into loop domains by binding to AT-rich sequences called matrix attachment regions (MARs) via nuclear matrix binding proteins. In HIV, >98% of HIV isolates have highly conserved MAR sequences spanning nt −350 to −150 relative to the transcription initiation site in the 5′ LTR (31). Tethering of the nuclear matrix protein SMAR1 at this site is associated with transcriptional repression of HIV (31). In accordance with previous findings, knockdown of SMAR1 in U87MG cells enhanced LTR activity by 1.9-fold under integrated conditions and by 1.8-fold when the LTR-luciferase construct was transiently transfected (Fig. 6a). SMAR1 siRNA efficacy was consistently ≥48% (data not shown). Given that the MAR sequence is located in the vicinity of the −143 site, we determined whether there is a relationship between TCF-4 binding to the HIV LTR and association with the nuclear matrix. SMAR1 was immunoprecipitated from the −143-spanning region in LTR-transfected astrocytes (Fig. 6b). Furthermore, deletion of the −143 TCF-4 binding site diminished SMAR1 binding to the LTR by approximately 50% (Fig. 6b), which suggests that this site is important for SMAR1 association with the HIV promoter. To determine whether TCF-4 directly interacts with SMAR1, we immunoprecipitated TCF-4 or SMAR1 from astrocytes and probed for SMAR1 or TCF-4, respectively. TCF-4 and β-catenin both coprecipitated with SMAR1, indicating that these proteins associate with each other *in vivo* (Fig. 6c, d, g, and h). SMAR1 band intensity was significantly diminished when the antibody was incubated with SMAR1-blocking peptide prior to Western blotting, demonstrating specificity of the SMAR1 antibody for its target (Fig. 6e and f).

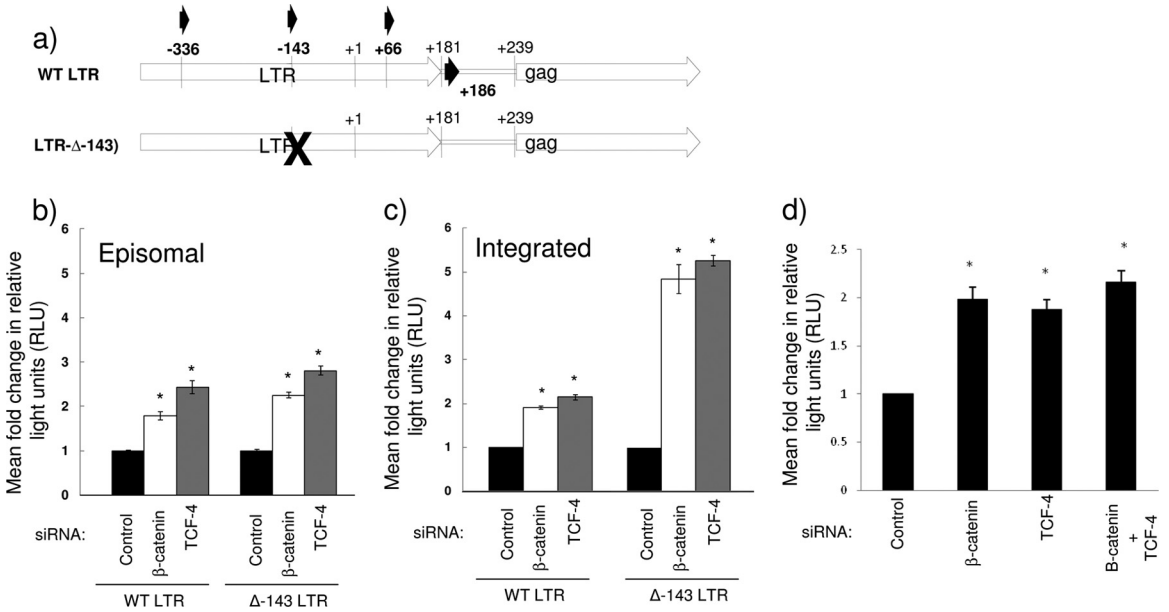


FIG 4 Deletion of TCF-4 binding sites at nt −143 enhances LTR activity, in combination with β-catenin/TCF-4 knockdown. (a) Schematic representation of HIV_{Bal} LTR constructs inserted into the pGL4.19 luciferase vector. The bold X denotes the deletion of the −143 site from the HIV LTR. (b) U87MG cells were transfected with the indicated siRNAs; 48 h later, the cells were transfected with WT LTR or Δ-143-LTR for an additional 24 h. The cells were lysed, and a dual-luciferase assay was performed. Readings were normalized to the total protein content as measured by BCA assay. (c) U87MG cells containing integrated WT LTR-luciferase or Δ-143-LTR-luciferase plasmid were transfected with the indicated siRNAs; 72 h postknockdown, the cells were lysed and a dual-luciferase assay was performed. Readings were normalized to the total protein content as measured by BCA assay. (d) U87MG cells containing integrated WT LTR-luciferase were transfected with control siRNA, β-catenin siRNA, TCF-4 siRNA, or both β-catenin and TCF-4 siRNA; 72 h postknockdown, the cells were lysed and a dual-luciferase assay was performed. Readings were normalized to the total protein content as measured by BCA assay. The data represent three independent experiments. *, $P < 0.05$ relative to control siRNA. The typical knockdown efficacy was >90%, as indicated previously (26). The error bars represent SD.

DISCUSSION

Considerable evidence from our laboratory and those of others indicates that the β-catenin/TCF-4 axis is a potent pathway for HIV suppression in multiple compartments, including astrocytes and peripheral blood lymphocytes (3, 20, 26, 28, 29, 38). The mechanism by which this pathway leads to HIV suppression is not entirely clear. We previously demonstrated that β-catenin and TCF-4 suppress HIV transcriptional activity in astrocytes (26) and

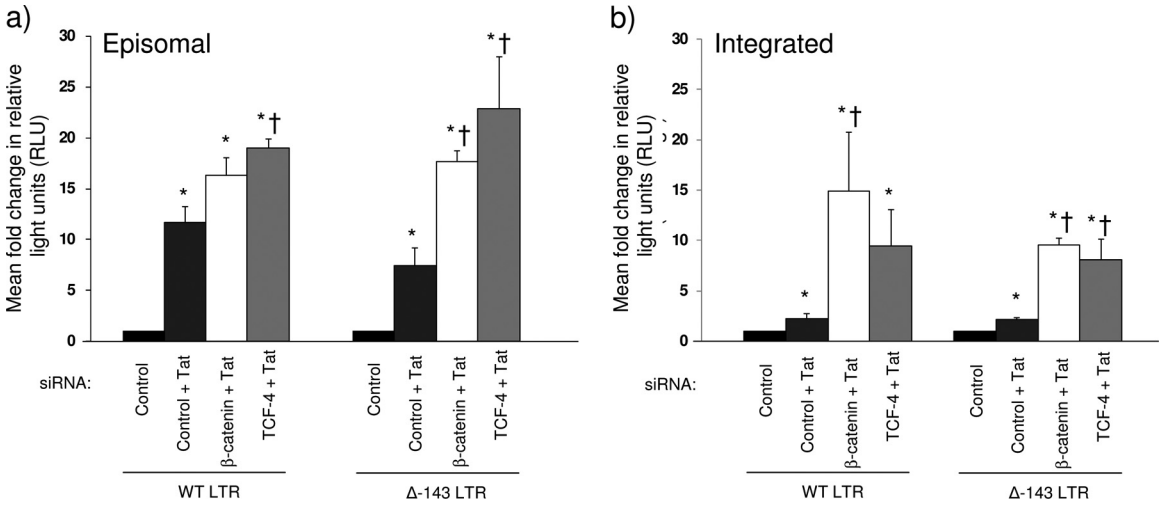


FIG 5 Knockdown of β-catenin or TCF-4 enhances Tat-mediated transactivation of the LTR independent of the nt −143 site. (a) U87MG cells were transfected with scrambled siRNA, β-catenin siRNA, or TCF-4 siRNA; 48 h postknockdown, the cells were transfected with WT-LTR or Δ-143-LTR luciferase constructs with either vector control (pcDNA3.1) or Tat expression plasmid for an additional 24 h. The cells were lysed, and a dual-luciferase assay was performed. (b) U87MG cells that stably express an integrated copy of the WT or a Δ-143-LTR-luciferase construct were transfected with the indicated siRNAs for 48 h, followed by transfection with vector control or Tat for an additional 24 h before the cells were lysed and a dual-luciferase assay was performed. Luciferase readings from panels a and b were normalized to the total protein content as measured by BCA assay. The data represent three independent experiments. *, $P < 0.05$ relative to control siRNA; †, $P < 0.05$ relative to control plus Tat. The error bars represent SD.

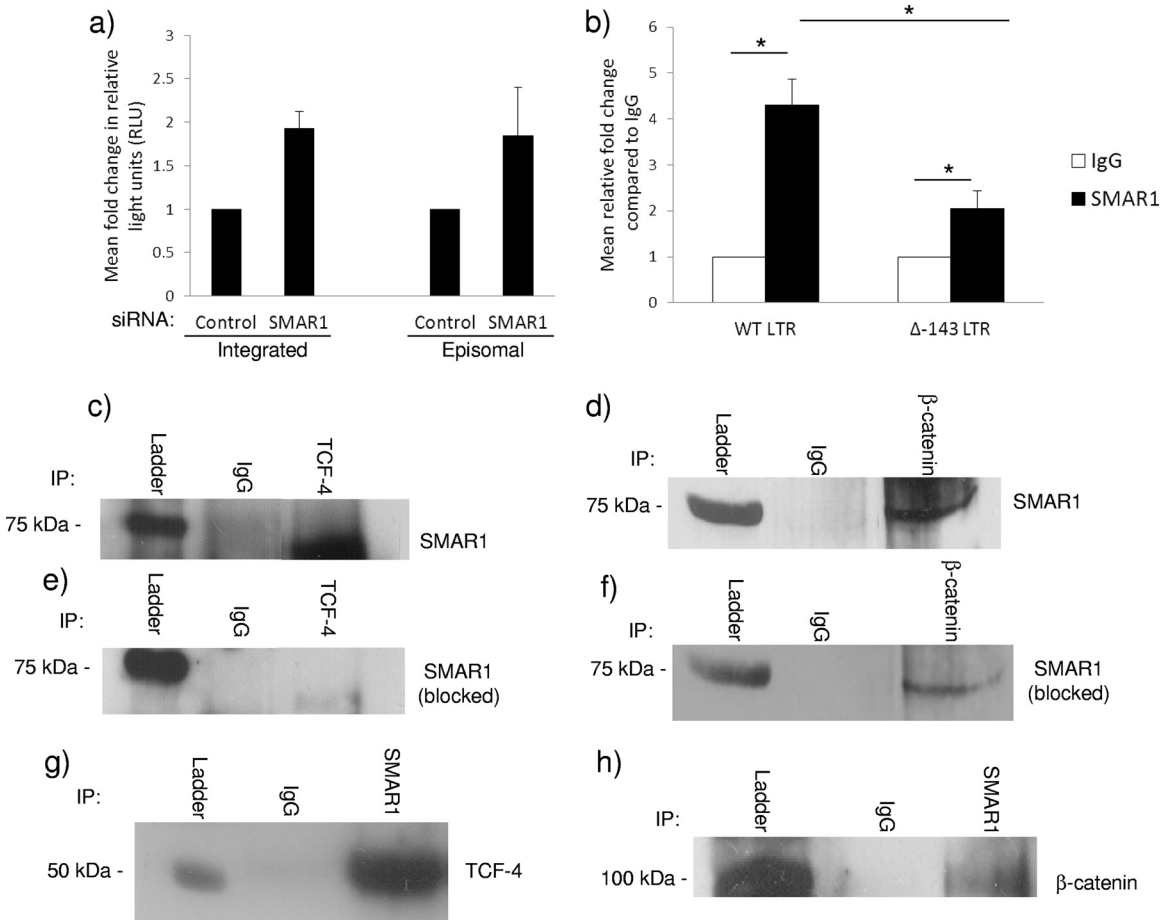


FIG 6 The nuclear matrix protein SMAR1 binds to the HIV LTR in astrocytes and associates with TCF-4 *in vivo*. (a) U87MG-LTR-luc containing an integrated HIV LTR-luciferase construct (integrated) or U87MG astrocytoma cells (episomal) was transfected with SMAR1 siRNA. After 48 h, U87MG astrocytoma cells were transfected with the LTR-luciferase construct for an additional 24 h. After 72 h of knockdown, all cells were lysed, and a luciferase assay was performed. Readings were normalized to the total protein content as measured by BCA assay. (b) U87MG astrocytoma cells were transfected with the indicated LTR-luciferase plasmid for 24 h. Chromatin immunoprecipitation was performed using antibodies to SMAR1 or rabbit IgG, followed by real-time PCR using primers encompassing nt −143, as described in Materials and Methods. (c to h) TCF-4, SMAR1, or β-catenin was immunoprecipitated from nuclear extracts generated from U87MG cells, followed by Western blotting to detect coimmunoprecipitated proteins, as indicated. For blocking experiments (e and f), SMAR1 antibody was incubated with a 5-fold excess of SMAR1-blocking peptide for 30 min prior to Western blotting to show the specificity of binding. The data are representative of three independent experiments. *, $P < 0.05$ relative to control. The error bars represent SD.

that knockdown of β-catenin or TCF-4 induced HIV promoter activity in astrocytes. As a DNA binding suppressor protein, TCF-4 may function in a number of ways, such as directly binding to the HIV LTR and interfering with RNA transcription machinery or bending the DNA away from a transcriptionally ready complex. This is a possibility, since TCF-4 contains HMG box domains that allow it to bind the minor groove and induce a sharp bend in the DNA helix.

Here, we assessed whether the HIV LTR has direct binding sites for TCF-4. We demonstrate that the promoter region of the HIV LTR contains four TCF-4 consensus binding sequences. At least two of these four sites were present in approximately 35% of HIV isolates examined. While Wortman et al. demonstrated that TCF-4 suppresses HIV transcription, they were unable to identify putative TCF-4 binding sites in the HIV LTR (38). This may be a reflection of the particular HIV strain they used to assess the presence of these putative sequences. We show that these TCF-4 binding sequences on the HIV LTR bind TCF-4 *in vivo* and have a higher affinity for binding at the nt −143 site. Further, our data indicate that these sites are widely present in the HIV LTR and that the kinetics of HIV replication and/or permissiveness for replication in astrocytes may be dependent on the HIV strain and whether it contains these TCF-4 sites.

Of the four TCF-4 binding sites identified in the HIV LTR, the nucleotide −143 site is of particular interest. This site is within 40 bp of the NF-κB binding site. Therefore, it may interfere with NF-κB binding. Indeed, some studies have indicated that active Wnt/β-catenin signaling may antagonize NF-κB activity (15), but these interactions may be cell type specific. We also demonstrated that knocking down TCF-4 enhances NF-κB binding to HIV LTR and its reporter activity (26). Deletion of the −143 site alone demonstrated a modest reduction rather than the anticipated enhancement of HIV promoter activity; however, this level of reduction is not likely to be biologically relevant. The relevance of the −143 site was clarified in our experiments utilizing stable cell lines that we generated to express WT LTR luciferase or a Δ-143-LTR

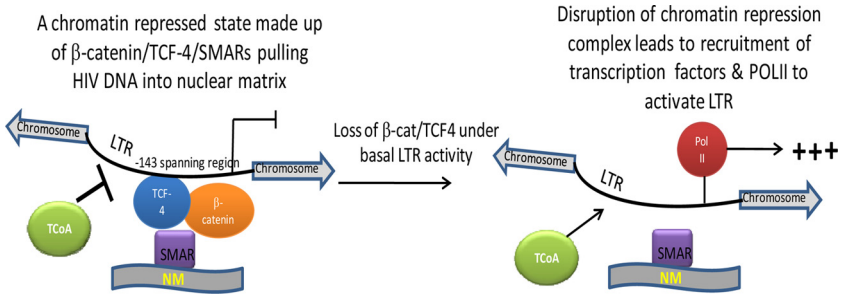


FIG 7 Proposed model of SMAR1/TCF-4/β-catenin repression of HIV transcription. Based on data presented here and by others (26), we propose that β-catenin/TCF-4/SMAR form a complex at nt −143 on the HIV LTR. This complex pulls HIV DNA spanning this region into the nuclear matrix and away from transcription machinery. Inhibition of β-catenin/TCF-4 by siRNA disrupts this chromatin repression complex and allows Pol II docking and recruitment of TCoA, such as NF-κB and C/EBP, to drive basal LTR activity.

luciferase construct. In the presence of siRNA for β-catenin or TCF-4, along with the Δ-143 construct, HIV LTR activity was markedly enhanced. It is possible that while deletion of the −143 site alone is not sufficient to enhance promoter activity, paired knockdown of β-catenin/TCF-4 leads to induction of transcriptional activators that removes a chromatin-repressed state to allow active transcription. This is especially significant because it suggests that the mechanism of β-catenin/TCF-4 repression of HIV may be multifaceted, including direct and indirect regulation of transcription. β-Catenin/TCF-4 affects other key transcriptional factors for HIV LTR activity. Knockdown of β-catenin/TCF-4 enhances binding of C/EBP family members and NF-κB to the HIV LTR (26).

Integrated HIV requires a certain level of basal transcription to accumulate Tat in order to transactivate the HIV LTR. HIV replication is greatly enhanced in the presence of Tat, largely due to increased processivity of RNA Pol II on the HIV promoter. Deletion of the −143 site had no impact on Tat transactivation of the HIV LTR, suggesting that this site is not directly involved in regulating Tat-mediated transcription. Nonetheless, knockdown of either β-catenin or TCF-4 greatly increased the ability of Tat to transactivate the LTR. Collectively, these data suggest that β-catenin/TCF-4 suppress Tat-mediated transactivation of the LTR in a manner that is independent of the TCF-4 binding site at nt −143 but may involve modification of a larger chromatin structure and/or negative regulation of other transcriptional enhancers required for HIV transcription.

The presence of a MAR in the vicinity of −143 prompted us to consider whether β-catenin/TCF-4 are involved in epigenetic silencing of HIV transcription through interactions with the nuclear matrix. Overexpression of the MAR binding protein SMAR1 potently represses HIV replication, likely through recruitment of the HDAC/Sin3 complex to the LTR (31). TCF-4 and β-catenin were immunoprecipitated with SMAR1 from astrocytes, and SMAR1 binding to the LTR was diminished by deletion of nt −143. These findings indicate that TCF-4/β-catenin/SMAR1 colocalize and suggest that TCF-4 and β-catenin are involved in SMAR1-mediated repression of HIV transcription. Although β-catenin/TCF-4 interaction is usually associated with transcriptional activation, there are now reports that this complex can also repress gene expression (16). Our findings strongly suggest that in the context of HIV, the association between β-catenin and TCF-4 leads to repression rather than induction of HIV promoter activity.

To our knowledge, this is the first study to provide evidence that TCF-4 and β-catenin are associated with the HIV promoter. Wortman and colleagues did not pursue a role for β-catenin in TCF-4-mediated repression of HIV replication because a dominant-negative form of TCF-4 that lacked the β-catenin binding site was able to repress LTR activity (38). In our hands, knockdown of β-catenin alone enhances HIV transcription in astrocytes (26), and we show here its association with other repressive complexes on the HIV LTR. Additionally, β-catenin may have another mechanism contributing to HIV inhibition beyond transcription. β-Catenin can also bind to cadherins at the cell membrane and may be involved in repression of HIV release (29). Specifically, the ability of HIV-encoded Vpu protein to enhance HIV release was linked to the ability of Vpu to reduce total β-catenin levels and interrupt β-catenin association with E-cadherins (10, 29). This finding is especially intriguing because it suggests that the association of β-catenin with E-cadherin inhibits the ability of Vpu to mediate viral-particle release.

Based on our findings, we propose a model in which β-catenin/TCF-4/SMAR form a complex at the −143 site of the HIV LTR (Fig. 7). This complex pulls the HIV DNA spanning this region into the nuclear matrix and away from the transcription machinery. Inhibition of β-catenin/TCF-4 by siRNA disrupts this chromatin repression complex and allows Pol II docking and recruitment of transcription coactivators (TCoA), such as NF-κB, NFAT, and C/EBP, to drive basal LTR activity (26). Therefore, signals that inhibit β-catenin/TCF-4 signaling are likely to induce HIV transcription by disrupting this inhibitory complex.

Infection of astrocytes or other cell types that have a significant level of endogenous β-catenin/TCF-4 signaling with HIV strains that contain TCF-4 sites in their LTR may be more susceptible to latent and/or low-level persistent HIV transcription rather than a productive/robust infection. Low-level HIV replication, particularly in an immune-privileged site such as the CNS, is a driving force in chronic inflammation and viral persistence. Even in the era of highly active antiretroviral therapy (HAART), it is estimated that ~50% of HIV-infected individuals develop some degree of HIV-associated neurocognitive disorders (HAND) (12). Moreover, at late stages of disease, the viral load in the CNS can exceed that in the periphery, and drug-resistant variants can occur at a greater frequency in the brain, possibly due to poor penetrance of therapeutics across the blood brain barrier (17). A greater understanding of the mechanisms involved in regulating low-level persistent HIV replication in cells such as astrocytes is warranted.

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