Interplay between viral Tat protein and c-Jun transcription factor in controlling LTR promoter activity in different human immunodeficiency virus type I subtypes

Renée M. van der Sluis,1 Ronald Derking,1 Seyguerney Breidel,1 Dave Speijer,2 Ben Berkhout1 and Rienk E. Jeeninga1

1Laboratory of Experimental Virology, Department of Medical Microbiology, Centre for Infection and Immunity Amsterdam (CINIMA), Academic Medical Centre, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands
2Department of Medical Biochemistry, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands

HIV-1 transcription depends on cellular transcription factors that bind to sequences in the long-terminal repeat (LTR) promoter. Each HIV-1 subtype has a specific LTR promoter configuration, and minor sequence changes in transcription factor binding sites (TFBSs) or their arrangement can influence transcriptional activity, virus replication and latency properties. Previously, we investigated the proviral latency properties of different HIV-1 subtypes in the SupT1 T cell line. Here, subtype-specific latency and replication properties were studied in primary PHA-activated T lymphocytes. No major differences in latency and replication capacity were measured among the HIV-1 subtypes. Subtype B and AE LTRs were studied in more detail with regard to a putative AP-1 binding site using luciferase reporter constructs. c-Jun, a member of the AP-1 transcription factor family, can activate both subtype B and AE LTRs, but the latter showed a stronger response, reflecting a closer match with the consensus AP-1 binding site. c-Jun overexpression enhanced Tat-mediated transcription of the viral LTR, but in the absence of Tat inhibited basal promoter activity. Thus, c-Jun can exert a positive or negative effect via the AP-1 binding site in the HIV-1 LTR promoter, depending on the presence or absence of Tat.

INTRODUCTION

The human immunodeficiency virus type I (HIV-1) can infect different cell types of the immune system, but preferentially targets proliferating CD4+ T lymphocytes. Once the viral DNA is integrated into the host cell genome, regulation of HIV-1 gene expression depends on the cellular transcription machinery. Transcription initiation is controlled by the viral promoter that is located in the U3 region of the long terminal repeat (LTR). The promoter encodes numerous transcription factor binding sites (TFBSs) that are bound by specific cellular transcription factors, which either induce or repress transcription (Pereira et al., 2000). The core promoter (−78 to −1, relative to the transcription start site at +1) plays a crucial role in HIV-1 transcription as deletion of the three SP1 sites, or the two NF-kB binding sites, severely impairs or even abolishes viral replication (Berkhout & Jeang, 1992; Kamine et al., 1991; Nabel & Baltimore, 1987; Ross et al., 1991). A low level of basal transcription is triggered by host TFs, but high levels of HIV-1 gene expression and subsequent virus production require the viral Tat protein. Tat interacts with the trans-activation-response region (TAR) element, an RNA motif located at the 5’ end of each HIV transcript (Berkhout et al., 1989). This interaction facilitates association of the positive transcription elongation complex-b (P-TEFb), which is composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (Marshall & Price, 1995). P-TEFb mediates hyperphosphorylation of the carboxy-terminal domain of RNA polymerase II, thereby strongly enhancing its elongation efficiency (Isel & Karn, 1999; Wei et al., 1998). If the HIV-1 promoter is not sufficiently active to support the production of a threshold amount of Tat protein, this can result in a latent HIV-1 provirus (Donahue & Wainberg, 2013; van der Sluis et al., 2013a; Van Lint et al., 2013).

Synthesis of the initial Tat protein molecules in an HIV-1 infected cell requires a basal level of transcription triggered by binding of cellular transcription factors to the LTR. Each HIV-1 subtype has a specific LTR promoter configuration and even minor sequence changes in TFBSs or their arrangement can strongly influence transcriptional activity, thereby affecting viral replication and latency properties (Crotti et al., 2007; Koken et al., 1992; Montano et al., 1991).
In a previous study, we constructed a set of isogenic viruses with subtype-specific promoter elements to investigate these functions (Centlivre et al., 2005, 2006; De Baar et al., 2000; Jeeninga et al., 2000; van der Sluis et al., 2011; van Opijnen et al., 2004a). Activation of a latent HIV-1 provirus in HIV-1-infected T-cell lines was triggered by TNFα, which activates the transcription factor NF-kB. We reported no gross differences among the subtypes, except for subtype AE that combines an increased level of basal transcription with a reduced TNFα response. This subtype AE-specific transcription profile was linked to the presence of a unique GABP binding site, instead of the regular NF-kB binding site, in the AE LTR. In this study, we tested the set of HIV-1 variants for their latency properties in primary effector T lymphocytes instead of T cell-lines. We further enhanced the study by investigating the subtype B and AE promoters in more detail, using reporter constructs with the LTR promoter driving the expression of the luciferase gene.

RESULTS AND DISCUSSION

HIV-1 subtype-specific latency and replication properties in primary T lymphocytes

We used recombinant viral genomes with subtype-specific promoters inserted in the common backbone of the subtype B LAI isolate (De Baar et al., 2000; Jeeninga et al., 2000). These recombinant LAI-A, -B, -C, -D, -AE, -F, -G and -AG viruses are isogenic except for the core promoter region that encodes the major TFBS, including two NF-κB sites (Fig. 1a). These subtype-specific viruses were previously tested for their latency properties in T-cell lines using a latex test following acute infection (van der Sluis et al., 2011). Here, we investigated these viruses in primary proliferating T lymphocytes. The HIV-1 provirus can be activated from latency in T-cell lines with TNFα or other drugs that activate the NF-kB TF (Duh et al., 1989; Duverger et al., 2009; Folks et al., 1989; Jeeninga et al., 2008; West et al., 2001; Williams et al., 2004). However, purging provirus in these primary T lymphocytes requires co-culturing of the HIV-1-infected T lymphocytes with dendritic cells (DCs) (van der Sluis et al., 2013b).

PHA-activated CD4+ T lymphocytes were infected with equal amounts (based on CA-p24 quantification) of the subtype-LTR viruses. Unbound virus was washed away after 4 h and the cells were cultured in the presence of the fusion inhibitor T1249 to prevent new rounds of infection. Cultures were split after 24 h into a mock treated culture and a DC co-culture. After another 24 h the cells were harvested, fixed, stained and analysed by flow cytometry for surface CD3 and intracellular CA-p24 expression. The mock treated culture yielded 2.3% CA-p24-positive cells for LAI-B (Fig. 1b). Similar results were obtained for the other subtypes. Following co-culturing with DCs, the percentage of CA-p24-positive cells increased significantly for all subtypes and the fold activation of HIV-1-producing cells was calculated (Fig. 1c). The percentage of CA-p24-positive cells increased approximately threefold for all subtypes, except for LAI-A. This subtype demonstrated a moderate twofold activation from latency, although the difference compared with other subtypes was not statistically significant. Thus, as previously described for subtype B, co-culturing of infected primary T lymphocytes with DCs activates this set of subtype-LTR viruses, but with similar efficiencies.

Viral replication properties can sometimes be linked to viral latency characteristics in T-cell lines. For example, subtype AE demonstrated reduced latency and replicates faster than subtype B in the SupT1 T cell line (van der Sluis et al., 2011; van Opijnen et al., 2004a). To investigate the influence of the subtype-specific promoter on viral replication capacity in primary T-cells, PHA-activated CD4+ T lymphocytes were infected with an equal amount of virus and replication was monitored by regular sampling of the culture supernatant for CA-p24 ELISA. Peak infection was reached 5 days post-infection for all subtypes (Fig. 2). The combined results show no gross differences among the subtypes in their latency properties and replication efficiency in primary effector T lymphocytes.

We previously measured reduced latency for subtype AE in SupT1 cells, but this phenotype was not apparent in primary cells. The observed difference between primary cells and the SupT1 T-cell line might be explained by NFκB levels. SupT1 T cells display low levels of active NF-kB under standard culture conditions and are therefore very responsive to NF-kB activation via TNFα stimulation (Chen et al., 1997). The primary cells were PHA activated prior to HIV-1 infection, which strongly activates the NF-kB pathway; thus, further enhancing this pathway with TNFα would have little effect. The AE LTR promoter is unique in that it contains a GABP site instead of the upstream NF-kB site, and is therefore less dependent on NF-kB activation, which may also explain the AE phenotype in SupT1 T cells. Activation of latent provirus in primary T cells by co-culture with DCs is triggered by a currently unknown signalling pathway, but probably does not involve NF-kB activation (van der Sluis et al., 2013b). Interestingly, subtype A displayed reduced activation from latency in primary cells, but did not exhibit a higher basal level of productive infection, suggesting either that this subtype establishes a smaller latent reservoir or that it was not efficiently purged from latency. Subtype A was recently reported to establish latent infections very efficiently in the Jurkat T-cell line (Duverger et al., 2013), supporting the idea that subtype A may be less efficiently purified.

Subtype-specific promoter characteristics

To study the promoter characteristics of the set of HIV-1 subtypes in more detail, luciferase reporter constructs with the subtype-specific LTR promoter were used (Jeeninga et al., 2000). HEK293T cells were transiently transfected with an LTR reporter (Fig. 3a) and a Renilla luciferase reporter plasmid to control for differences in transfection
efficiency. We measured no significant differences for the different subtype LTRs in basal promoter activity, which was arbitrarily set at unity for each subtype to compare LTR induction among the subtypes (Fig. 3b). To investigate NF-kB-induced LTR induction in the absence of Tat, the transfected cells were stimulated with TNFα. TNFα induction ranged from a modest fourfold activation for the subtype AE promoter to 12-fold for the subtype D promoter, with a mean ninefold activation for the subtype B promoter. Co-transfection with a Tat expression plasmid, without TNFα stimulation, induced the subtype B promoter 7.8-fold, while the other subtype LTRs showed induction ranging from fivefold for subtype AG to 14.5-fold for subtype AE. TNFα treatment in the presence of Tat further boosted the LTR promoter activity for all subtypes, with the lowest (13-fold) induction observed for subtype AG and the highest (36-fold) for subtype B. Thus, TNFα increased the basal promoter activity of all subtypes at a statistically
significant level, but with a relatively low induction for subtype AE, as expected due to the lack of a second NF-kB site. Tat induction, on the contrary, is higher for AE compared with the other subtypes. Interestingly, subtype C, which was predicted to have three NF-kB sites (Fig. 1a), responded similarly to TNFα treatment as the other subtype LTRs with the regular two NF-kB sites.

The GABP binding site

Substitution of the regular upstream NF-kB site with a GABP site in subtype AE determines its unique transcriptional profile of reduced latency combined with increased basal transcription (Jeeninga et al., 2000; van der Sluis et al., 2011). We therefore created new B and AE reporters in which these TFBSs were exchanged (Fig. 4a). Luciferase assays indicated that the B and B + GABP reporters exhibit lower basal activity than the subtype AE and AE + 2 × NFkB reporters, but the differences were not statistically significant (Fig. 4b). The basal promoter activity was arbitrarily set at unity to compare the induction properties. Adding the GABP site to the subtype B LTR indeed reduced TNFα induction and increased Tat induction (Fig. 4c). Conversely, adding the NF-kB site to AE restored TNFα induction and decreased Tat induction.

The putative AP-1 binding site

Another prominent difference between the B and AE promoters is a putative AP-1 binding site that partially overlaps the RBEIII binding site (Fig. 1a) (Jeeninga et al., 2000). ‘AP-1 transcription factor’ is a collective term for a group of structurally and functionally related proteins that can bind to a DNA consensus sequence known as the TPA-responsive element (TRE; 5′ TGA(C/G)TCA 3′) (Meng & Xia, 2011). AP-1 is involved in a broad range of cellular processes. Each of these proteins is differentially expressed in different cell types, meaning that every cell type has a unique profile of AP-1 dimers (Hess et al., 2004).

To study the influence of this AP-1 binding site, mutations were created that either disrupt the AP-1 site in the subtype AE LTR (AE–AP1) or introduce the AP-1 site in the subtype B LTR (B + AP1, Fig. 5a). The mutations were designed not to affect the conserved upstream RBEIII binding site but may have affected the TFII-I site that

![Fig. 2](https://example.com/f2.png)

**Fig. 2.** HIV-1 subtype-specific replication in primary T lymphocytes. Viral replication of the indicated subtype-specific viruses in primary T lymphocytes was monitored by regular sampling of culture supernatant for CA-p24 ELISA. Results are obtained from experiments with two different donors, performed in duplicate.

![Fig. 3](https://example.com/f3.png)

**Fig. 3.** HIV-1 subtype-specific promoter characteristics in HEK293T cells. HEK293T cells were transfected with HIV-1 subtype-specific LTR luciferase reporter constructs (schematically shown in a) with and without co-transfection of the Tat-expressing plasmid or TNFα treatment (b). To compare the different reporters, the basal promoter activity (without Tat and TNFα) was arbitrarily set at unity. Results are obtained from four independently performed experiments and each experiment was performed in triplicate. Statistical significance levels are indicated: 0.05 (*) and 0.01 (**).
contributes to basal LTR promoter activity (Dahabieh et al., 2011; Koken et al., 1994; Malcolm et al., 2008; Sadowski & Mitchell, 2005). The B+AP1 reporter consistently showed a slightly higher, though not statistically significant, basal promoter activity than the B reporter (Fig. 5b), and the basal promoter activities for the AE2AP1 and wild-type AE LTR reporters were comparable, suggesting no major effects of the mutations on the TFII-I site in this setting.

To compare induction of the LTR reporters, the basal promoter activity was set at unity. TNFα-mediated LTR induction decreased from 10.5-fold for B LTR to sixfold for B+AP1 (Fig. 5c). Introducing the AP-1 site in the subtype B LTR also slightly decreased Tat induction, from 5.9- to 4.2-fold. Disruption of the AP-1 site in the subtype AE promoter did not affect TNFα and Tat responses.

The LTR constructs were next examined in a Tat titration experiment. Induction of the wild-type B LTR was apparent with increasing amounts of Tat, while B+AP1 remained mostly unaffected (Fig. 5d). Tat equally enhanced transcription from the AE and AE−AP1 LTRs (Fig. 5e). Thus, the introduction of AP-1 to the B LTR increased basal promoter activity, but decreased Tat induction. The equivalent mutations in subtype AE had little effect.

Expression of c-Jun

To investigate whether the AP-1 site is targeted by an AP-1 transcription factor, the reporter constructs were co-transfected with a vector encoding c-Jun, which belongs to the AP-1 family of transcription factors. Expression of the c-Jun protein was demonstrated by Western blotting of cell extracts (Fig. 6a). Overexpression of c-Jun decreased basal promoter activity (i.e. without Tat) of the AE reporter (Fig. 6b). Surprisingly, the promoter activity of subtype B also decreased (although this was somewhat less pronounced), even though it lacks the AP-1 consensus sequence. c-Jun expression decreased B+AP1 promoter activity more than that of the wild-type B LTR promoter. As expected, transcription of the AE LTR lacking the AP-1 site (AE−AP1) was hardly influenced by c-Jun overexpression. These results indicate that c-Jun inhibits the
basal promoter activity of both the subtype B and AE LTR, but the consensus AP-1 site as present in subtype AE potentiates the suppression.

To investigate whether c-Jun also inhibits LTR promoter activity in the presence of Tat, the reporter constructs were co-transfected with the c-Jun expression vector and the Tat expression vector. Results are obtained from four independent experiments, each experiment being performed in triplicate. Statistical significance levels are indicated: 0.05 (*) and 0.01 (**).

**Fig. 5.** Subtype AE contains a putative AP-1 binding site. (a) Schematic of the promoter elements in the LTR of subtypes B and AE. Indicated are TFBSs for RBEIII, AP-1, NF-κB, GABP and Sp1. (b) HEK293T cells were transfected with the B and AE LTR luciferase constructs as indicated, and basal promoter activity was determined by the firefly/Renilla ratio. (c) HEK293T cells were transfected with the B and AE LTR reporters with and without co-transfecting the Tat expressing plasmid or TNFα treatment. For comparison, basal promoter activity (without Tat and TNFα) was arbitrarily set at unity. (d) HEK293T cells were transfected with the wild-type (B) or mutant (B + AP1) LTR reporters and increasing amounts of the Tat expression vector. (e) HEK293T cells were transfected with the wild-type (AE) or mutant (AE – AP1) LTR reporters and increasing amounts of the Tat expression vector. Results are obtained from four independent experiments, each experiment being performed in triplicate. Statistical significance levels are indicated: 0.05 (*) and 0.01 (**).
expression vector. c-Jun overexpression had no effect on Tat-induced induction of the subtype B LTR reporter (Fig. 6c). Surprisingly, c-Jun increased Tat-mediated induction of the B + AP1, AE and AE–AP1 reporters. Thus, c-Jun enhances Tat-mediated LTR transcription but suppresses basal LTR transcription without Tat.

**AP-1 and the different HIV-1 subtypes**

Next, the other subtype LTRs were tested for c-Jun responsiveness. Like subtype AE, promoters of subtype C, G and AG are predicted to have a single AP-1 site, and subtypes A and F are predicted to have two (Fig. 7a) (Jeeninga et al., 2000). HEK293T cells were transfected with an LTR reporter with and without the c-Jun expression plasmid. To compare the c-Jun effect, the basal activity of each LTR was arbitrarily set at unity. Although there was variation among the subtypes, c-Jun decreased the basal promoter activity of all LTR reporters (Fig. 7b). However, the variation in c-Jun response could not be linked to the number of predicted AP-1 sites.

To test the effect of c-Jun in the presence of Tat, the reporters were co-transfected with the c-Jun and Tat expression vectors. As expected, c-Jun expression did not further enhance promoter activity of the 'AP-1-free' subtype B LTR in Tat-expressing cells (Fig. 7c). The Tat-induced promoter activity of the LTR reporters with the AP-1 binding site(s) (A, C, AE, F, G and AG) increased in the presence of c-Jun. Again, the variation in c-Jun response could not be linked to the number of AP-1 sites. Somewhat surprisingly, Tat-mediated induction of the subtype D LTR, which does not seem to have an AP-1 site, also increased in the presence of c-Jun and Tat when compared with Tat alone. This may be explained by the fact that although the subtype D LTR does not fully match the proposed AP-1 consensus, it forms a better match than the subtype B LTR, allowing the D LTR to be selectively induced by c-Jun in the presence of Tat. In conclusion, without Tat, c-Jun inhibits basal transcription for the different subtype LTRs. In the presence of Tat, c-Jun enhances transcription from all LTRs except subtype B, degrading this from the 'subtype prototype' to the 'odd one out'.

The dual response of c-Jun overexpression on LTR transcription needs further research to be fully understood. c-Jun preferentially heterodimerizes with the AP-1 family member c-Fos but can also form protein complexes with members of the ATF and MAF protein families or homodimerize with other c-Jun proteins, making the AP-1 signalling network very complex (reviewed by Eferl & Wagner, 2003). Individual AP-1 proteins display different transcriptional activation potential: c-Jun, c-Fos and FosB proteins are generally considered strong activators, while JunB and JunD are considered weak (Hess et al., 2004). Under certain conditions, JunB and JunD proteins can act as repressors of AP-1 activity by competing for binding to TRE sites, or by forming ‘inactive’ heterodimers with c-Jun, c-Fos or FosB, adding an extra layer to the already complex AP-1 network. A previous study, which used the same reporter constructs used here, demonstrated that there is a delicate balance between the different AP-1 proteins with regard to induction of the LTR promoter. Pertussis toxin B-oligomer increased FosB, Fra2 and ATF2 protein levels, and this correlated with decreased LTR promoter activity (Rizzi et al., 2006). This was only observed for constructs containing the predicted AP-1 binding site. In this study we increased c-Jun protein levels, which directly influenced LTR promoter activity, but this may indirectly have affected endogenous levels of c-Jun.
**Fig. 7.** AP-1 and the different HIV-1 subtypes. (a) Schematic of the TFBSs in the different HIV-1 subtype LTRs. Indicated are the RBEIII, AP-1, NF-KB, GABP and Sp1 sites. (b) HEK293T cells were transfected with the subtype-specific LTR reporters with and without co-transfection of the c-Jun expression plasmid (as indicated). (c) Transfection of HEK293T cells with the different indicated LTR reporters with and without the Tat and/or c-Jun expression plasmid (2 ng). Results are obtained from three independent experiments, each performed in triplicate. Statistical significance levels are indicated: 0.05 (*) and 0.01 (**).
Our experiments show that the HIV-1 LTR is responsive to Tat protein changes, and the AP-1 binding site is an important determinant of viral latency as part of the latency establishment element. In this transcription study, c-Jun suppressed LTR activity in the absence of Tat, and c-Jun may thus contribute to the establishment of a latent provirus. Interestingly, c-Jun overexpression instead boosts LTR promoter activity in the presence of Tat. In other words, c-Jun provides the setting for Tat to act as a molecular switch of HIV-1 LTR activity that is both sensitive and dominant.

**Concluding remarks**

Our experiments show that the HIV-1 LTR is responsive to the c-Jun protein. This effect is strengthened when the LTR contains an AP-1 site that matches the TRE consensus more closely, as in subtype AE. Duverger et al. (2013) recently published a study proposing that the AP-1 binding site is an important determinant of viral latency as part of the latency establishment element. In this transcription study, c-Jun suppressed LTR activity in the absence of Tat, and c-Jun may thus contribute to the establishment of a latent provirus. Interestingly, c-Jun overexpression instead boosts LTR promoter activity in the presence of Tat. In other words, c-Jun provides the setting for Tat to act as a molecular switch of HIV-1 LTR activity that is both sensitive and dominant.

**METHODS**

**Cells.** The human embryonic kidney cell line HEK293T was grown as a monolayer in Dulbecco's minimal essential medium supplemented with 10% (v/v) FCS, 40 U penicillin ml⁻¹, 40 μg streptomycin ml⁻¹ and non-essential amino acids (Gibco) at 37 °C and 5% CO₂. For the isolation of CD4⁺ primary T lymphocytes and monocytes, human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Central Laboratory Blood Bank, Amsterdam, The Netherlands) by use of a Ficoll gradient. Monocytes were subsequently isolated with a CD14 selection step using a magnetic bead cell sorting system (Miltenyi Biotec). Purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated into DCs by stimulation with 45 ng interleukin-4 ml⁻¹ (Miltenyi Biotec). Purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated into DCs by stimulation with 45 ng interleukin-4 ml⁻¹ (Miltenyi Biotec). Purified monocytes were cultured in RPMI 1640 medium containing 10% FCS and differentiated into DCs by stimulation with 45 ng interleukin-4 ml⁻¹ (Miltenyi Biotec).

**Reagents.** TNFα (PHC3015; Invitrogen) was prepared in sterile MilliQ H₂O (stock solution 10 μg ml⁻¹) and used at a final concentration of 10 ng ml⁻¹. The fusion inhibitor T1249 (WQWEQKITA-LLEQAQIQQKENYELQDKLWASLWEWF) was obtained from Pepscan and used at a final concentration of 0.1 μg ml⁻¹.

**Antibodies.** For intracellular CA-p24 FACS staining we used z-CA-p24-RD1 (clone KC57; Coulter). For CD3 staining, the APC-conjugated z-CD3 (BD Bioscience) was used. For DC staining, purified z-CD83-APC (BD Bioscience), z-CD86-PE (BD Pharmingen), z-HLA-DR-PerCP/Cy5.5 (BD Bioscience), z-CD14-FITC (BD Bioscience) and z-DC-SIGN-PE (R&D Systems) antibodies were used. For protein detection on Western blot the mouse monoclonal anti-β-actin (Sigma), rabbit polyclonal anti-c-Jun (Abcam), goat anti-rabbit or goat anti-mouse HRP-labelled secondary antibodies (KPL) were used.

**Plasmids.** LTRs from patient isolates representing subtypes A, C, D, AE (CRF_01), F, G and AG (CRF_02) were selected as being representative of the viral quasi-species in the patient and the HIV-1 subtypes (De Baar et al., 2000). The BseAI-AfII fragment (position 2147–163) of the LTR was exchanged in an LTR-luciferase plasmid that is based on the subtype B LAI sequence (Jenninga et al., 2000). Introduction of the GABP in place of the upstream NF-KB site in the promoter of subtype B and the conversion of the unique GABP in AE into a second NF-KB site in the full-length molecular clone pLAI (Peden et al., 1991) have previously been described (van der Sluis et al., 2011; Verhoef et al., 1999). To introduce the B+GABP and AE+2×NF-KB LTRs in the luciferase reporter constructs, the LTR was excised from pLAI with Xhol-Bgl restriction enzymes and cloned into pBlue3LTR (Jenninga et al., 2000). From this intermediate plasmid the LTR was excised by restriction with BseAI-AfII and cloned into the LTR-luciferase plasmid using these sites.

To introduce the AP-1 site in the subtype B promoter, pBlue3’LTR B was used as a template in two independent PCRs under standard conditions. PCR primers 5’ GAA CTG CTG ACA AAG AAG TTG CTA C 3’ and standard primer 1 (5’ TGT CTC ATG AGC GGA TAC ATA 3’) were used in reaction A (bold indicates the AP-1 site). Reaction B was done with primers 5’ CTT CTT CGA CAG TCC TTG AAG TAC 3’ and standard primer 2 (5’ TGG AAG GGC TAA TTC ACT CTC 3’). Both PCR products, purified from gel, were used as templates in a third PCR under standard conditions with standard primers 1 and 2. The 833 bp PCR product was digested with BseAI and HindIII, purified from gel and ligated into pBlue3’LTR that was previously cut with the same enzymes. To remove the AP-1 site from the AE promoter, pBlue3’LTR AE (Jenninga et al., 2000) was used as a template in two independent PCRs under standard conditions. PCR primers 5’ GAC TCG TGA CAT CGA AGT TTC TCA C 3’ and standard primer 1 were used in reaction A (bold indicates the mutated AP-1 site). Reaction B was done with primers 5’ CTT CGA TGT CAG TCT TTA TAG TAC 3’ and standard primer 2. Both PCR products, purified from gel, were used as templates in a third PCR under standard conditions with standard primers 1 and 2. The 833 bp PCR product was digested with BseAI and HindIII, purified and ligated into pBlue3’LTR that had previously been cut with the same enzymes. The mutated LTRs were cloned from the pBlue3’LTR vector into the luciferase plasmid using the BseAI-AfII restriction sites, or into the full-length molecular clone pLAI with Xhol-Bgl restriction sites. All new constructs were verified by sequencing.

The p Tat exon contains the Tat codon sequence under control of the constitutive cytomegalovirus (CMV) promoter (Verhoef et al., 1997). To control for transfection efficiency, the pRL-TK (GenBank accession number AF025846) plasmid (Invitrogen) is used. In this plasmid the Renilla ORF is under the constitutive expression of the TK promoter that is not affected by TNFα treatment as the promoter does not have an NF-KB binding site.

For c-Jun expression, the full-length cDNA clone of the JUN gene (IRAT97080488D) was excised from pBlueScript II (BioScience) with EcoRI-Apol restriction sites and ligated into the pCMV-Sport6 (Invitrogen) expression plasmid. The pCMV-Sport6-cJun expression plasmid was verified with digestion restriction analysis and sequencing.

**Luciferase assay.** DNA transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In short, 2.5 × 10⁶ HEK293T cells were seeded 24 h prior to
transfection in a 96-well plate. The following day, cells were transfected with a luciferase reporter plasmid (1 ng) and pRL-TK (3 ng) in the presence or absence of pcDNA3-Tat vector (1 or 3 ng) and/or pCMV-Sport6-c-Jun (2 or 4 ng). To equalize the total amount of DNA for transfection, the empty pcDNA3 vector was used, and to normalize for transfection efficiency, Renilla expression from pRL-TK was used. For the c-Jun overexpression experiments, cells were transfected with 3 ng pcDNA3-Tat vector, and for the TNFz-induction cells were transfected with 1 ng pcDNA3-Tat vector. For TNFz treatment, cell culture medium was replaced 18 h post-infection with mock medium or medium supplemented with 10 ng ml−1 TNFz. Cells were harvested 24 h post-transfection and luciferase activity was measured with the DualGlo Luciferase kit (Promega). All transfections were performed in triplicate. Results from different assays were corrected for between-session variation with the factor correction program (Ruitter et al., 2006).

**Western blot.** HEK293T cells (0.5 x 10⁶) were seeded 24 h prior to transfection with the pCMV-Sport6-c-Jun using Lipofectamine 2000 (Invitrogen). The cells were collected 24 h post-transfection, lysed by dissolving the cells in Laemmli sample buffer and heated at 95 °C for 10 min. Proteins were separated on SDS-PAGE and transferred onto an Immobilin_P membrane (Millipore). Blots were blocked and incubated overnight with the primary antibody followed by incubation with HRP-labelled secondary antibodies. Luminometric detection of proteins was performed with Western Lightning ECL (PerkinElmer Life Sciences) and membranes were analysed on a LAS3000 imager (GE Healthcare).

**Virus production and replication.** Plasmid DNA encoding the CXCR4-using HIV-1 LAI primary isolate (Peden et al., 1991) or derivatives thereof were transiently transfected into HEK293T cells with the calcium phosphate method as described previously (Das et al., 1999). Virus supernatant was harvested 2 days after transfection, sterilized by passage through a Whatman 0.2 μm filter (GE Healthcare) and stored in aliquots at −80 °C. The concentration of virus stocks was determined by CA-p24 ELISA. To study viral replication, CD4⁺ T lymphocytes isolated from two different blood donors were pooled and 1.0 x 10⁶ cells were infected with the different isogenic viruses (1 ng CA-p24 for each virus). Supernatant samples of infected cultures were taken on different days for extracellular CA-p24 analysis.

**Extracellular CA-p24 ELISA.** Culture supernatant was heat inactivated at 56 °C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem). The CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom) as capture antibody, and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as detection antibody. Quantification was performed with a twin-site ELISA with D7320 (Biochrom) as capture antibody, and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as detection antibody. Quantification was performed by a twin-site ELISA with D7320 (Biochrom) as capture antibody, and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as detection antibody. Quantification was performed

**FACS flow cytometry.** Cells were fixed in 4% formaldehyde for 10 min at room temperature and subsequently washed with FACS buffer (PBS supplemented with 1% FCS). The cells were permeabilized with BD Perm/Wash buffer (BD Pharmingen), and antibody staining was performed in BD Perm/Wash for 1 h at 4 °C. Excess of unbound antibody was removed and the cells were analysed on a BD FACSCanto II flow cytometer with BD FACSdiva Software v6.1.2 (BD Biosciences) in FACS buffer. The live population was defined based on forward/sideways scatter and analysed for CD3 and intracellular CA-p24 positivity. Gate settings were fixed between samples for each experiment. The DC phenotype [negative for CD14, low levels of MHC class II (HLA-DR), CD83 and CD86 and high levels of DC-SIGN] was confirmed by FACS flow cytometry (Sanders et al., 2002).

**ACKNOWLEDGEMENTS**

We thank S. Heijnen for performing CA-p24 ELISA and J. A. Dobber for maintenance of the BD FACSCanto II. Research by R. v. d. S. and R. E. J. was supported by the Dutch AIDS Fund (AIDS Funds 2007028 and 2008014; http://www.aidsfonds.nl/about/organisation).

**REFERENCES**


