# HIV-1 gene expression: characterization of viruses with altered transcription elements

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# HIV-1 gene expression: Characterization of viruses with altered transcription elements

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# HIV-1 GENE EXPRESSION: CHARACTERIZATION OF VIRUSES WITH ALTERED TRANSCRIPTION ELEMENTS

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aan mijn ouders,
voor Mara

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# Chapter 1

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# Introduction

## 1. The retrovirus HIV-1

### 1.1 General overview of retroviral replication

Retroviruses are RNA viruses that use a DNA intermediate in their life cycle. The RNA genome of retroviruses is relatively small (approximately 10 kb) and contains the *gag*, *pol* and *env* open reading frames encoding the structural proteins and enzymatic functions that are unique for this group of viruses. Retroviral particles are enveloped by a lipid membrane and have a diameter of approximately 100 nm. The *env*-encoded glycoproteins that mediate adsorption to susceptible cells are present at the surface of the virus particle. The virion core consists of several proteins encoded by the *gag* gene and contains the essential *pol* enzymes protease, reverse transcriptase and integrase. Two copies of full-length genomic RNA are co-packaged into the virion core and form a dimer. Retroviral infection of a cell is initiated by interaction between the envelope glycoprotein on the virus particle and host cell receptors. The membrane of the virion fuses with the cellular membrane and the virion core is released into the cytoplasm. The viral RNA is copied into double-stranded DNA by the viral reverse transcriptase enzyme within this core structure. The core migrates to the nucleus and the DNA is integrated in the host genome at a random position by the viral integrase enzyme. The integrated provirus subsequently serves as a template for the production of RNA by the transcription machinery of the host cell. The RNA encodes the viral proteins or functions as genomic RNA that is packaged into the virion. Virus particles assemble at the cell membrane and are released by budding, after which they mature by processing of the gag and gag-pol precursors proteins by the virus-encoded protease enzyme. An extensive overview of retroviral replication can be found elsewhere (34).

### 1.2 Classification of HIV-1

Seven genera of retroviruses have been defined on the basis of genome structure and nucleotide sequence relationships (34). The Lentivirus genus consists of the Visna/Maedi Virus, Caprine Arthritis-Encephalitis Virus (CAEV), Equine Infectious Anemia Virus (EIAV), Bovine

Immunodeficiency Virus (BIV), Feline Immunodeficiency Virus (FIV), the Simian Immunodeficiency Viruses (SIV) and the Human Immunodeficiency Viruses type 1 and 2 (HIV-1 and HIV-2). These viruses cause several neurological or immunological disorders in their mammalian hosts and a characteristic is that disease progression is generally slow.

### 1.3 HIV-1 tropism and pathogenesis

HIV-1 has been recognized as the infectious agent that causes Acquired Immune Deficiency Syndrome (AIDS) in humans. The related HIV-2 also causes AIDS, but disease progression may be slower compared with HIV-1. HIV replicates in CD4-positive cells ($CD4^+$), including monocytes, macrophages and T lymphocytes and enters cells through an interaction of the Env glycoprotein of the virion with the CD4 receptor and specific chemokine receptors on the target cell membrane (8,52,75,100,101). In general, HIV-1 isolates can infect either monocytes and macrophages or T lymphocytes, although some dual-tropic isolates have been described (46). A primary determinant for this differential tropism lies within the variable V3 domain of the Env glycoprotein (162). The viral Env protein interacts preferentially with the CCR5 chemokine receptor expressed by monocytes and macrophages, or the CXCR4 receptor present on T-lymphocytes.

The course of HIV infection is characterized by an acute and a steady state phase. The virus replicates to high titers after primary infection, coinciding with a rapid decline of CD4-positive cells (108). This acute infection is eventually controlled by an HIV-specific immune response, that leads to a sharp decrease in viral load and a restoration of the $CD4^+$ cell count. Following this relatively short period of acute infection, there is a much longer period of clinical latency, in which $CD4^+$ lymphocyte count drops slowly and in which a gradual increase in viral load is observed. In recent years, a large body of evidence has been generated that demonstrates continuous viral replication during this period. It was demonstrated that HIV-1 infection in this steady state involves the production and destruction of billions of virus particles and $CD4^+$ cells per day (74,134,178). Despite a vigorous cellular and humoral immune response, the infection is not cleared and the immune system eventually gets exhausted, making the individual vulnerable to opportunistic infections.

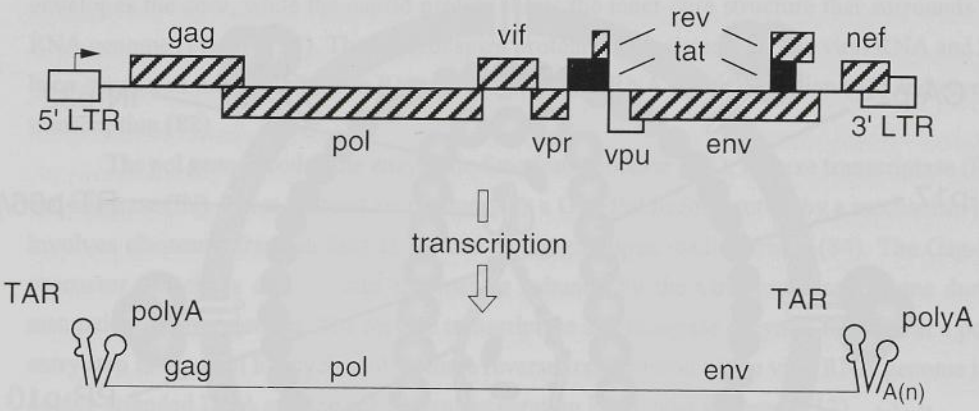


Figure 1: The HIV-1 genome.

The double-stranded DNA proviral genome is flanked by Long Terminal Repeats (LTRs), of which the 5' LTR acts as a promoter for the synthesis of viral RNA (the start site of transcription is indicated by an arrow). The positions of the HIV-1 open reading frames are indicated. The *trans*-acting proteins Tat and Rev are encoded on two exons and regulate HIV-1 gene expression. Two important RNA structure motifs, TAR and polyA, are schematically drawn at the termini of the viral transcript.

## 1.4 HIV-1 genome structure

The proviral genome of HIV-1 is around 9.5 kb in length and, like all retroviruses, contains genes for the Gag, Pol and Env gene products (Fig 1). The integrated provirus is flanked by two identical DNA sequences termed the Long Terminal Repeats (LTR's). These direct repeats can be subdivided in three regions: U3, R (repeat) and U5 that play distinct roles in viral transcription and reverse transcription. HIV-1 transcripts contain a copy of the R region at either end, but the U3 and U5 sequences are uniquely present at the 3' and 5' end of the RNA, respectively. A complex mechanism of reverse transcription is required to regenerate the complete LTR sequences at both ends of the provirus.

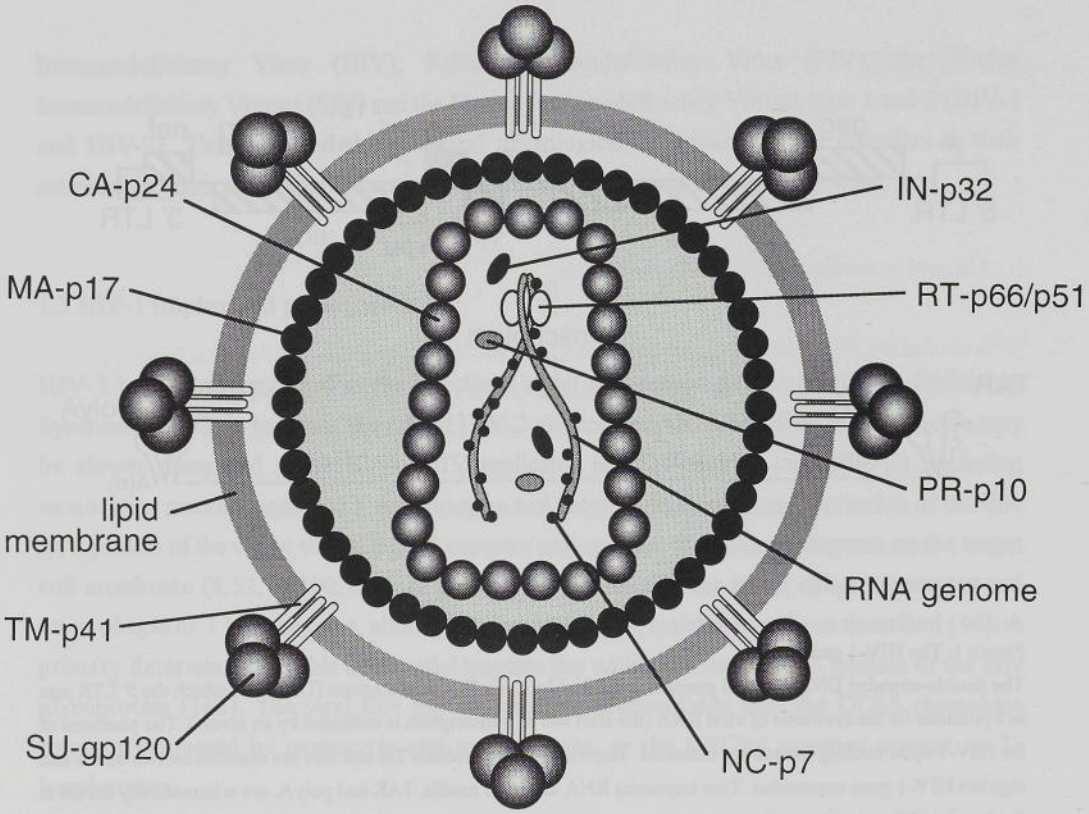


Figure 2: Structure of the HIV-1 virion particle.
The lipid membrane that envelopes the virus particle contains the Env glycoprotein products SU-gp120 and TM-p41. The *gag*-encoded matrix proteins (MA-p17) line the inside of the lipid membrane, and the capsid (CA-p24) proteins form the cone-shaped virion core. The *pol*-encoded products integrase (IN-p32), protease (PR-p10) and reverse transcriptase (RT-p66/p51) are present within this core structure, together with two copies of the viral RNA genome and the associated nucleocapsid (NC-p7) proteins.

## 1.5 HIV-1 proteins

The matrix (MA), capsid (CA) and nucleocapsid (NC) proteins that are encoded by the *gag* gene are translated as a Gag polyprotein. After assembly of the virus particle, proteolytic processing of this precursor protein by the viral protease enzyme yields the mature proteins that form the

core structure of the virion. The matrix protein is associated with the lipid membrane that envelopes the core, while the capsid protein forms the inner core structure that surrounds the RNA genome (Fig 2) (113). The nucleocapsid protein is associated with the viral RNA and has been suggested to be involved in RNA packaging (19), RNA dimer formation (43) and reverse transcription (82).

The pol gene encodes the enzymatic functions protease (PR), reverse transcriptase (RT) and integrase (IN). These proteins are produced as a Gag-Pol fusion protein by a mechanism that involves ribosomal frameshifting at the end of the *gag* open reading frame (84). The Gag-Pol precursor protein is cleaved into the mature subunits by the viral protease enzyme during maturation of virus particles. The reverse transcriptase and integrase enzymes function at a post-entry step in the viral life cycle and mediate reverse transcription of the viral RNA genome into double-stranded DNA and the subsequent integration in the host genome (113).

HIV envelope proteins are expressed at the surface of the virus particle and mediate the attachment to specific cell receptors (CD4 and chemokine coreceptors) and fusion of the viral membrane with the cell membrane. The envelope glycoprotein is expressed as a 160 kDa protein that is co-translationally translocated into the endoplasmatic reticulum (E.R.) by means of an N-terminal signal sequence. In the E.R., the signal peptide is removed and the protein is extensively glycosylated, specific disulfide bridges are formed and the protein forms trimers. In the Golgi apparatus, the gp160 protein is cleaved into a surface (SU-gp120) and a transmembrane part (TM-gp41) that remain associated through non-covalent interactions, and the proteins are transported to the cell membrane (161).

In addition to the basic set of retroviral genes described above, HIV-1 encodes several accessory viral functions that play a role in different steps of the virus life cycle: Vif, Vpr, Vpu and Nef. Although none of these proteins are absolutely required for HIV-1 replication, cell-type specific contributions to virus production and infectivity have been described (for a detailed discussion about the function of these proteins, see (164)). The essential Tat and Rev regulatory proteins regulate HIV-1 gene expression at the transcriptional and post-transcriptional level, respectively, and are discussed in more detail below.

## 2. HIV-1 gene expression

HIV-1 gene expression is dictated by a complex interplay between *cis*-acting viral DNA/RNA sequences, viral transactivator proteins and host cell proteins. Transcription is controlled by the

promoter that is located in the U3/R region of the 5' LTR. Since the proviral 5' and 3' LTR are identical in sequence and both contain the required signals to promote transcription, the question arises whether the 3' LTR is also functional as a transcriptional promoter. RNA transcribed from the latter promoter will read into chromosomal sequences downstream of the integrated provirus. That the 3' LTR of a retrovirus can function as a promoter was discovered in studies of retroviral-induced activation of proto-oncogenes. However, it appeared that the transcriptional activity of the 3' LTR promoter (expressing the proto-oncogene) is dependent on deletion or inactivation of the 5' LTR (56,125,133). A study on the promoter function of the HIV-1 5' and 3' LTRs confirmed the results obtained with avian retroviruses. The 3' LTR was active as a promoter, most notably upon inactivation of the upstream 5' LTR, although absolute levels of transcription remain much reduced compared with the functional 5' LTR promoter (103). Because the 5' LTR promoter will produce elongation complexes that can prevent or disrupt the assembly of initiation complexes at the downstream 3' LTR (39,103), this mechanism was termed transcriptional interference or promoter occlusion (3).

Transcription is executed by the cellular RNA polymerase II (RNA pol II) enzyme and the transcription efficiency is dramatically enhanced by the viral Tat transactivator protein.
The mechanism of regulated viral transcription is discussed in detail in section 3. The full-length transcript is around 9.5 kb in size and functions both as genomic RNA and messenger RNA for the synthesis of Gag and Gag-Pol precursors. This transcript is also the precursor for the more than 30 differentially spliced, subgenomic mRNA species that encode the other viral proteins. Spliced HIV-1 messenger RNA is translated in the cytoplasm to produce the regulatory proteins Vif, Vpr, Tat, Rev, Vpu and Nef and the structural Env glycoprotein. Splicing is executed by the host cell splicing machinery, but this process is regulated by the viral Rev protein (see below).

Like most cellular mRNAs, HIV-1 transcripts are capped at the 5' end and polyadenylated at the 3' end (136). Capping involves the enzymatic addition of a cap group (m7G5'pppN) to the most 5' nucleotide of the viral RNA, which is at the base of the TAR hairpin structure (Fig 4). Capping is important for recognition of the RNA by components of the cellular translation machinery (108). Polyadenylation of the viral RNA is also executed by host cell enzymes. The AAUAAA (polyA) signal that directs polyadenylation at the 3' end of the RNA is present in the R region of the viral RNA and is thus repeated at both the 5' and the 3' end of the transcript. Polyadenylation at the 5' site will yield a short, non-coding RNA and will reduce the amount of full-length viral RNA. To prevent premature polyadenylation, HIV-1 has developed a regulatory mechanism which involves occlusion of the polyA signal in a stable RNA structure (42,99). This

suboptimal setting allows both complete suppression of the 5' signal due to 5' specific inhibitory elements and full activity of the 3' signal due to 3' specific enhancer elements (42).

The primary HIV-1 transcript contains splice signals that can be used by the splicing machinery to generate one of the more than 30 different HIV-1 mRNA species. HIV-1 splicing is regulated by the affinity of the different splice signals for components of the splicing machinery (for a review, see (136)). Early after infection, multiply spliced transcripts are produced predominantly, which encode the regulatory Tat, Rev and Nef proteins. Expression of the viral Rev protein shifts the balance toward the appearance in the cytoplasm of singly spliced and unspliced RNAs that encode the structural Gag, Pol and Env proteins. Rev interacts with an extensively folded RNA motif in the *env* coding region, termed the Rev-responsive element (RRE). Binding of Rev to the RRE initiates the multimerization of Rev on the RNA and promotes nuclear export of RRE-containing (unspliced or singly spliced) RNA (136).

## 2.1 HIV-1 Transcription

Viruses are obligatory parasites and are optimally adapted to use the host cell machinery for the production of new virus particles. This is also true for HIV-1 and the LTR promoter contains *cis*-acting regulatory signals that are recognized by host cell transcription factors. Thus, HIV-1 transcription is executed by the same enzyme system that expresses cellular genes, that is the cellular RNA polymerase II (RNA pol II). We will first provide a brief overview of the mechanism of eukaryotic transcription and subsequently discuss the HIV-1 promoter including the TAR motif (section 3.1) and the Tat transactivator protein (section 4) that control viral gene expression.

The basic factors required for RNA pol II transcription described below have been identified in reconstituted *in vitro* transcription reactions with purified protein fractions and represent a minimal transcription system. Transcription *in vivo* is much more complex and requires a large number of cofactors in addition to those described below. At promoters that are transcribed by RNA pol II, assembly of the pre-initiation complex at the TATA-box is started by an interaction of the TATA-box binding protein (TBP) with the DNA. TBP associates with TBP-associated factors (TAF's) to form the TFIID complex (26). Subsequently, transcription factor TFIIB binds the TFIID-DNA complex and recruits the RNA pol II holoenzyme with its associated TFIIF complex (25). The TFIIE and TFIIH transcription factors also bind to the initiation complex and mediate the melting of the DNA duplex around the start site of transcription (76). This open complex allows for the synthesis of the first phosphodiester bonds. The TFIIH enzyme

is thought to be involved in the subsequent process of promoter clearance, either through its helicase activity (63) or by phosphorylation of the C-terminal domain of RNA pol II by the TFIIH-associated kinase (CDK7) (5). Promoter clearance marks the switch from transcription initiation to elongation, and the basal transcription factors leave the elongating RNA pol II complex, with the exception of TFIIF. General transcription elongation factors such as P-TEFb promote efficient transcription and prevent the RNA pol II complex from arrest (138). Transcription termination is coupled to 3' end processing, which involves cleavage of the transcript and poly(A) addition (65). Recent reports indicate that transcription by RNA pol II and pre-mRNA processing are linked in a number of ways, since essential subunits for mRNA capping, splicing and polyadenylation associate with the RNA pol II enzyme during transcription (reviewed in (127)).

## 3 The HIV-1 LTR promoter

The HIV-1 promoter is located mainly in the U3 region of the LTR and contains a large number of *cis*-acting sequences that control basal and activated transcription. Part of this region also encodes the Nef protein that is essential for viral replication in primary cell types. Transcription starts at the U3/R border, such that the R-U5 sequences form the 5' end of the RNA. The downstream R-U5 region also contains elements that are important for HIV-1 transcription. One transcription motif is active as part of the viral transcript: the extreme 5' end of the messenger RNA folds a hairpin structure termed TAR (*trans*-acting response region) that forms the binding site for the viral Tat transactivator protein (see below).

The HIV-1 core promoter maps to sequences -80 to -1 (relative to the start site of transcription) and contains the TATA box (position -28 to -24) and three binding sites for the Sp1 transcription factor (positions -78 to -45). The TATA box serves as the assembly site for the transcription initiation complex and positions the RNA pol II complex to start transcription at the U3/R border.

The ubiquitously expressed Sp1 transcription factor is required for basal LTR transcription, as was determined by mutational analysis of the Sp1 binding sites (17,67,91). In addition, Sp1 was shown to be essential for Tat-mediated activation of transcription from the HIV-1 promoter (15,17) and directly interacts with Tat *in vitro* and *in vivo* (88).

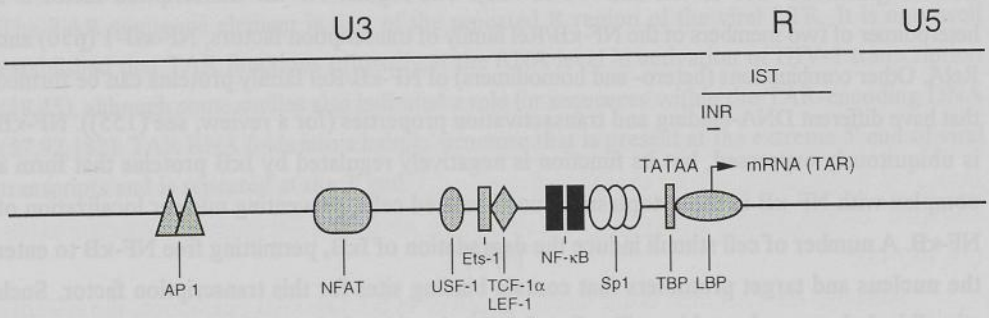


Figure 3: Transcription elements in the HIV-1 LTR promoter.
The U3, R and U5 regions of the LTR and binding sites for several transcription factors, as well as other sequence elements are indicated (see text for further details). The arrow at the U3-R border denotes the start site of transcription.

The spacing between the Sp1 sites and the HIV-1 TATA box is critical for optimal Tat transactivation and thus provides functional evidence for a role of Sp1 in Tat-mediated transactivation of the HIV-1 promoter (80).

Another factor that interacts with sequences in this region is the LBP-1 protein. Three binding sites for this factor are present at the U3-R border around the start site of transcription (-17 to +22). In the same area, a regulatory element has been described that directs the synthesis of short transcripts in the absence of the Tat transactivator protein (inducer of short transcripts (IST); (151)). This region also contains sequences that resemble an initiator (Inr) element, a non-TATA DNA element that can trigger transcription and that for instance is present in the promoter of the terminal transferase gene (141). The role of the IST and Inr elements in HIV-1 transcription is not well understood and analysis of these elements is obscured by the presence of overlapping elements that regulate transcription (such as TAR).

The upstream U3 region of the HIV-1 LTR that is positioned upstream from the core promoter (positions -454 to -80) contains binding sites for a large number of transcription factors, that either positively or negatively regulate HIV-1 transcription. The most well-studied factor that

binds to sequences in this region is NF-κB, for which two binding sites are present in most HIV-1 isolates (positions -105 to -96 and -91 to -82). The regular NF-κB transcription factor is a heterodimer of two members of the NF-κB/Rel family of transcription factors, NF-κB-1 (p50) and RelA. Other combinations (hetero- and homodimers) of NF-κB/Rel family proteins can be formed that have different DNA-binding and transactivation properties (for a review, see (155)). NF-κB is ubiquitously expressed, but its function is negatively regulated by IκB proteins that form a complex with NF-κB in the cytoplasm of unstimulated cells, preventing nuclear localization of NF-κB. A number of cell stimuli induce the degradation of IκB, permitting free NF-κB to enter the nucleus and target promoters that contain binding sites for this transcription factor. Such stimuli include several cytokines, T-cell activation signals and physical/chemical stress.

HIV-1 transcription is dependent on the integrity of the NF-κB binding sites in the U3 enhancer, as was determined in subgenomic transfection experiments (17,123). In these analyses, NF-κB elements were shown to be required for basal promoter activity and dispensable for Tat-activated transcription. Studies on the role of the NF-κB elements in HIV-1 replication either do (7,31) or do not (107,142) support an essential role for these enhancer elements in virus growth.

The promoter-distal U3 region contains binding sites for several transcription factors that are commonly found in T lymphocyte nuclei. Most of these binding sites are not highly conserved among viral isolates, and their importance for HIV-1 gene expression is still largely unknown. Starting from the 5' end of the U3 enhancer region, two binding sites for the AP-1 (a fos/jun heterodimer) and/or COUP transcription factors are present at positions -357 and -324 (35,112,129). Binding sites for the NFAT transcription factor are present at positions -254 to -216. USF-1 is able to bind to a site between positions -173 to -159 (157). The region between nucleotides -149 to -141 functions as a binding site for the Ets-1 transcription factor (156). A sequence immediately downstream from this position (-143 to -122) is recognized by the TCF-1α/LEF-1 transcription factor (153). Numerous cooperative interactions between the above mentioned factors have been described, and further testing of replicating virus in different cell types is required to learn more about these interactions within the HIV-1 enhancer region. Deletion studies have demonstrated a small negative effect of the upstream enhancer region on HIV-1 transcription (111). No effect on replication was measured in a study where the upstream enhancer region of the SIV LTR was extensively mutagenized, leaving the *nef* coding potential intact (83).

### 3.1 The *trans*-acting responsive (TAR) RNA element

The TAR sequence element is part of the repeated R region of the viral LTR. It is now well established that TAR functions primarily at the RNA level in activation of HIV-1 transcription (18,45), although some studies also indicated a role for sequences within the TAR-encoding DNA (57,92,182). TAR RNA folds into a hairpin structure that is present at the extreme 5' end of viral transcripts and is repeated at the 3' end.

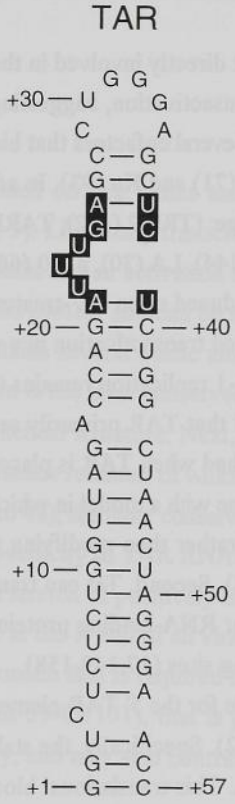


Figure 4: The TAR RNA element.

RNA secondary structure model of the TAR element present in the R region of all HIV-1 transcripts. The three-nucleotide bulge domain forms the binding site for the Tat protein, and the nucleotides that are crucial for the Tat/TAR interaction are shaded. The six-nucleotide loop forms the binding site for cyclin T, the cellular factor that is involved in Tat-mediated transactivation through the TAR motif. Nucleotides are numbered from the start site of transcription (+1).

In the 5' context, TAR serves as the binding site for the viral Tat protein and cellular cofactors in the process of transcriptional activation (18,45). TAR is transcriptionally active as part of the nascent transcript, which has important implications for the mechanism of Tat-mediated transactivation (18). The 5' TAR RNA hairpin is formed by basepairing of sequences between position +1 and + 57 relative to the start site of transcription (Fig 4). Important features of TAR are the highly conserved 3-nucleotide pyrimidine bulge in the stem and the apical 6-nucleotide loop. Tat binds specifically to the bulge domain of TAR (reviewed in (163), see below) and mutation of the bulge or the flanking basepairs abolishes Tat-mediated transactivation (14,16,145).

Although the TAR loop is not directly involved in the Tat-TAR interaction, the integrity of this domain is important for Tat transactivation, suggesting that a cellular cofactor recognizes these unpaired nucleotides (51,145). Several cofactors that bind to this loop have been identified: p68 (115), TRP-185 (181,183), p83 (71) and Ku (93). In addition, numerous factors have been identified that bind either the TAR bulge: (TRP-2 (152), TARBP-b (137), BBP (10)), or the TAR stem: (TRBP (59), SBP (143), PKR (144), LA (30), p140 (66), RNA pol II (184)). Furthermore, at least 7 TAR-binding factors are induced upon UV-treatment of cells (28). Although some of these factors do stimulate Tat-mediated transactivation in a variety of transcriptional assays, the significance of these factors for HIV-1 replication remains to be established.

Several studies demonstrated that TAR primarily serves to recruit Tat to the promoter. First, transactivation by Tat is abolished when TAR is placed further downstream of the HIV-1 transcription initiation site. This is in line with a model in which the Tat-TAR complex loops back to activate events at the promoter, rather than modifying the actively elongating RNA pol II complex to which it is attached (148). Second, Tat can transactivate the HIV-1 LTR promoter when fused to heterologous DNA- or RNA-binding proteins and recruited to the promoter via the corresponding DNA/RNA binding sites (17,149,158).

Several studies indicated a role for the 5' TAR element in regulation of translation of the HIV-1 mRNAs (21,22,37,132,150,172). Specifically, the stable TAR RNA structure was shown to inhibit efficient mRNA translation. This translational block was overcome in the presence of the Tat transactivator protein. Several mechanistic explanations have been postulated to explain this inhibitory effect. For instance, the TAR hairpin may block ribosomes that are scanning the mRNA. Alternatively, the stable TAR hairpin may occlude the cap structure and thus inhibit the binding of translation initiation factors (132). TAR was also shown to activate the double-stranded RNA-dependent kinase PKR, which phosphorylates and inactivates eIF-2, a factor that is essential for translation initiation (150).

Apart from its role in transcription and translation, the TAR element has also been described to be involved in other steps of the viral life cycle (102). Specifically, both the 5' and 3' TAR hairpins contribute to optimal packaging of the viral transcript into virions (41,120), but most 5' TAR mutants exhibit a transcription defect, and part of the reduction in virion RNA is likely to be caused by reduced HIV-1 RNA levels in the cell (41,171). A role for TAR in reverse transcription was reported in one study (69). However, it may be that the observed reduction of reverse transcription products following infection of cells with HIV-1 TAR mutant viruses can be fully attributed to a decreased genomic RNA content of the mutant virus particles (41).

## 4 The HIV-1 Tat protein

The Tat protein of HIV-1 is encoded on two exons and consists of 86 to 101 amino acids, depending on the viral isolate (Fig 5). Like many transcriptional activators, Tat has a modular structure, with an RNA-binding domain and an activation domain. The latter domain is encoded within the first 48 amino acids of the protein and can be subdivided into three regions. The N-terminal region of 21 residues contains several acidic amino acids and proline residues and is termed the acidic domain. This region is the least conserved part of the activation domain and is predicted to form an amphipathic α-helical structure. Next, the cysteine-rich region (residues 22 to 37) contains 7 highly conserved cysteine residues, of which 6 are critical for Tat function (147). The central core region (residues 38 to 48), the most conserved domain of Tat, is an essential part of the activation domain and adds specificity to TAR RNA-binding (33,170). The RNA-binding domain (residues 49-58) consists of a stretch of positively charged amino acids that interact with the TAR RNA hairpin that is present at the 5' end of all viral transcripts (175). Part of this motif also forms the nuclear localization domain that is required to direct Tat to the nucleus (72,160). The C-terminal part of Tat (residues 59-86/101), that is partially encoded on a second exon, contributes marginally to Tat activity, and may also contribute to RNA-binding (33,168).

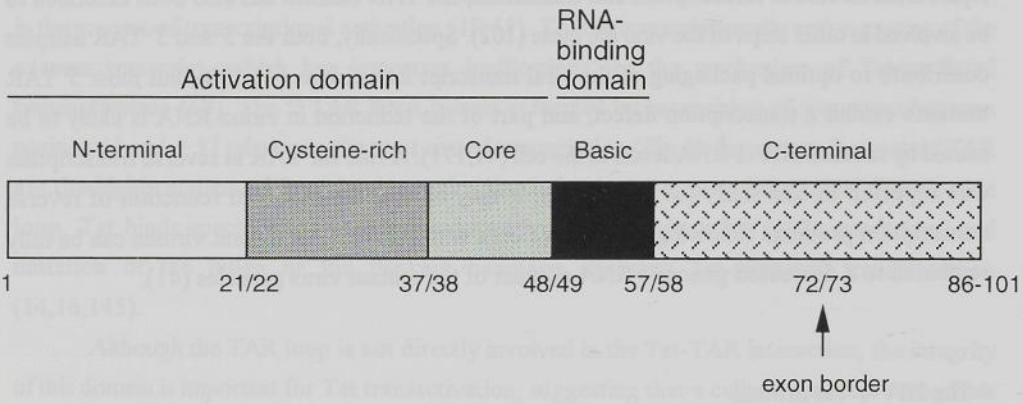


Figure 5: The Tat domain structure

The Tat domain organization was proposed on the basis of several mutational studies, as well as phylogenetic comparison of Tat proteins from different lentiviruses (105). The N-terminal 48 amino acids form the activation domain and putatively interacts with the transcription machinery. The basic domain, that mediates binding to the TAR RNA element, also functions as a nuclear localization signal. The C-terminal domain, that is partially encoded by the second coding exon of Tat, contributes marginally to Tat function.

The second coding exon of Tat encodes the C-terminal 14-29 amino acids. The strong conservation of the second Tat coding exon suggests an important function in the viral life cycle. Several studies proposed specific roles for the second coding exon of Tat: transactivation of chromosomally integrated LTR promoters (87), interaction with PKR (121), downregulation of MHC class 1 expression (78) and entry of extracellular Tat into cells (RGD motif) (23). It has been suggested that virus isolates that encode a 86 amino acid Tat protein have been adapted to growth on transformed T lymphocytes, and that full-length Tat (101 residues) is required for optimal virus replication *in vivo* (128). However, a stopcodon at the end of the first coding exon (position 72) was observed in several natural isolates of SIV, suggesting that the second Tat coding exon is not essential for virus replication (89). Therefore, the presence of an early stopcodon at codon position 87 in some virus isolates may represent natural variation rather than adaptation of the virus to growth in tissue culture (128,168) and is possibly dictated by the overlapping *env* and *rev* sequences.

## 4.1 Structural studies on Tat

A typical feature of the Tat protein of all HIV/SIV isolates is a cluster of cysteine residues in the activation domain. Mutational studies demonstrated the importance of this cysteine-rich domain for Tat function (105,140,146). Though the primary sequence of this part of the activation domain does not predict a classical zinc-finger motif, zinc was demonstrated to form a complex with Tat in a 2:1 molar ratio (53,104). A structure model of Tat was presented, in which two Tat molecules cooperatively coordinate four zinc ions (53). However, the biological function of zinc-binding was not addressed in this study. The binding of Zinc to Tat was confirmed in another study, but this interaction was found to inhibit Tat function and it was proposed that the essential cysteine residues of Tat are involved in intramolecular disulfide bridge formation (104).

Biophysical studies on the Tat protein revealed little structural detail sofar (54,64,110,122). This type of analysis is frustrated by the poor solubility properties of Tat and the readily oxidizing cysteine residues in the activation domain. The best resolution NMR picture obtained sofar is provided by Paul Rosch and coworkers from the University of Bayreuth (12). The core domain and the C-terminal domain of Tat contain stable secondary structure and are surrounded by flexible loops of the cysteine-rich and basic domains.

## 4.2 Tat-binding proteins

Transactivation of the HIV-1 promoter by Tat requires cellular factors, and a large number of proteins has been identified that interact with Tat. For instance, Tat was found to bind the Sp1 transcription factor (88), RNA polymerase II (119), TBP (TFIID) (97), TAP (187), a zinc-finger protein (55), TAK (73)/P-TEFb (177), TBP-1 (126), p36 (44), MSS1 (154), TIP30 (186), Tip60 (95), p300/CBP (77,118) and TAFII250 (179). Although some of the interactions were demonstrated to be functional in transcription assays, e.g. Tat binding to Sp1, p300/CBP and TAK/P-TEFb, no conclusive data has been provided on the role of these interactions during a natural HIV-1 infection.

## 4.3 The Tat/TAR interaction.

The interaction of Tat with the TAR RNA element was first analyzed using *in vitro* binding assays with Tat and TAR mutants. These analyses revealed epitopes on Tat and TAR that mediate the interaction. Tat binds to TAR via a stretch of basic amino acids, termed the basic domain (residues 49-58) in a 1:1 complex. This Tat domain interacts with nucleotides of the highly conserved TAR bulge domain (Fig 4). Nuclear Magnetic Resonance (NMR) spectroscopy studies on the free TAR RNA reveal that this hairpin is rather flexible, especially in the bulge area (1,2). The triple pyrimidine bulge distorts the RNA helix and introduces a bend in the RNA, thereby opening the major groove. It is thought that this widened groove allows for hydrogen-bonding between TAR residue G26 and Arg52 of Tat (24,27). Other positively charged amino acids in the basic domain of Tat possibly interact with the negatively charged phosphate backbone of the RNA, thereby increasing the RNA-binding affinity (176). Amino acid sequences flanking the basic domain also contribute to the affinity of Tat for TAR (33). Binding of Tat to TAR induces a conformational shift that straightens the RNA helix and stabilizes the TAR structure (130).

## 4.4 Putative additional functions of Tat

In addition to its role in regulation of HIV-1 transcription, Tat has been suggested to function in other steps of the virus life cycle, to affect expression of cellular genes of both infected and uninfected cells and to induce apoptosis and T cell anergy .

A role of Tat in stimulation of translation of HIV-1 messenger RNAs was suggested by several groups (see section 3.5). In a study that employed complementation of transcription of a Tat-minus virus by other viral transactivators, it was suggested that Tat has a role in virion infectivity (81). Similarly, it was described that virions of a Tat-defective HIV-1 mutant are defective in reverse transcription and that this defect could be complemented by Tat (68).

Tat influences the expression of several cellular genes, most notably the expression of cytokines (reviewed in (29)). For example, Tat was implicated in the downregulation of the MHC class 1 promoter (78), but this effect was found to be transient and limited (168). In addition, Tat decreases the production of C-C chemokines that inhibit HIV-1 replication (188), and increases the expression of chemokine receptors that mediate infection by HIV-1 (79).

HIV-1 Tat is actively secreted from cells by a still unidentified mechanism that does not require the presence of a signal sequence (50) and exerts a pleiotropy of effects on neighboring

cells. Tat has been proposed to bind the integrin receptors via an RGD peptide motif encoded by the second *tat* exon. However, this motif is absent in several HIV-1 Tat sequences, and trans-cellular effects were demonstrated to be independent of the second coding exon (169). Tat was shown to promote the growth of spindle cells derived from Kaposi Sarcoma, an angioproliferative disease frequently observed in AIDS patients (6,11,49). Also, extracellular Tat was able to induce or sensitize cells to undergo apoptosis (109,180).

Several reports link HIV-1 Tat protein to perturbation of the immune response. For instance, Tat may induce the generation of suppressor cells in populations of uninfected T cells, that downregulate the immune response (188). This report was preceded by a study that demonstrated Tat-mediated inhibition of antigen-induced lymphocyte proliferation (173). Two other observations also suggest a role for Tat in HIV-1 pathogenesis. First, high anti-Tat antibody titers are correlated with long term survival of HIV-1 infection (139). Second, Tat-specific CTL frequencies inversely correlate with progression to AIDS (165). Further studies are required to confirm these findings on the role of Tat in dysregulation of the immune system.

### 4.5 Mechanism of Tat action

The identification of the stage of transcription at which Tat exerts its function has generated much debate. Depending on the system used, effects were found at an early step (transcription initiation) or a later step (transcription elongation/anti-termination), or both (20,86,87,96,106). It is likely that the Tat effect on transcription initiation is largely dependent on the level of basal LTR transcription (98). In systems in which the LTR promoter directs a high basal level of transcription, Tat mainly stimulates transcription elongation, and no increase in initiation of transcription can be measured. For instance, the HIV-1 LTR directs a high basal level of transcription in *in vitro* reconstituted transcription reactions, and no increase in transcription initiation is measured upon addition of Tat protein in this system (86,116,131). Similarly, in transient transfection assays of COS cells with LTR reporter constructs containing the SV40 origin of replication, basal transcriptional activity of the LTR is dramatically amplified through replication of the reporter plasmid (96,98). In these artificial assay systems, Tat acts predominantly at the level of transcriptional elongation.

The *in vivo* situation, in which a single integrated provirus is transcribed, may be much more complex. Although the basal promoter activity of an integrated HIV-1 provirus may depend on the site of integration (124), basal transcription levels in this setting are characteristically low,

and rate-limiting steps different from those observed in *in vitro* transcription reactions may apply (87). Experiments that employ such integrated templates demonstrate that transactivation by Tat has a predominant effect on transcription initiation (87). An alternative explanation for an effect of Tat on transcription initiation could be that Tat may indirectly stimulate transcription initiation by increasing promoter clearance, since promoter clearance in one round of transcription can facilitate initiation complex formation in the next (185).

### 4.6 Tat interacts with a CTD kinase that phosphorylates the RNA pol II C-terminal domain

A well-studied factor that controls elongation of transcription in mammalian cells is the RNA polymerase II (RNA pol II) holoenzyme itself. The C-terminal domain (CTD) of the largest RNA pol II subunit contains 52 repeats of the Tyr-Ser-Pro-Thr-Ser-Pro-Ser motif. Phosphorylation of the serine, threonine and tyrosine residues in these repeats is correlated with the elongation capacity of the enzyme (40). The unphosphorylated form of the RNA polymerase (RNA pol IIa) is associated with paused polymerase complexes, whereas the phosphorylated form (RNA pol IIo) is found in elongation-competent RNA pol II complexes.

It was demonstrated that the requirement of the CTD in transcription by RNA pol II is promoter-dependent (60). The HIV-1 LTR promoter, unlike for instance the HTLV-1 LTR promoter, was found to depend on the integrity of this RNA pol II domain for activated transcription (32). This study also demonstrated that Tat associates *in vitro* with a kinase activity that can phosphorylate the C-terminal domain of RNA pol II. It was further shown that the kinase inhibitor staurosporine was able to reduce CTD phosphorylation and Tat-mediated activation of transcription in *in vitro* assays. Interestingly, Tat transactivation of the HIV-1 promoter through TAR was also found to be sensitive to treatment with the purine nucleoside analog 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB) (116). In particular, the formation of long transcripts by elongation-competent RNA polymerase II complexes was inhibited. DRB, like staurosporine, inhibits kinases that can phosphorylate the RNA pol II CTD. In addition, all compounds that were identified in a screen for inhibitors of Tat transactivation contained kinase inhibitory activity (114). It was therefore postulated that Tat recruits a kinase to the promoter that phosphorylates the CTD of RNA pol II to stimulate transcription elongation.

Two kinases have been implicated in transcriptional activation of the HIV-1 LTR promoter by Tat/TAR: the cyclin-dependent kinases CDK7 and CDK9. CDK7 is associated with cyclin H in the CAK and TFIIH complexes (90). CDK9 (TAK or PITALRE) was recently shown to

interact with cyclin T in the human P-TEFb complex (177). Using Tat column chromatography purification of HeLa nuclear extract, a protein fraction was isolated with CTD kinase activity. This fraction was shown to contain several TFIIH components and increased Tat-mediated activation of transcription in *in vitro* reactions (58) The TFIIH complex is involved in an early step of transcription, that is the formation of the open complex and/or promoter clearance (63). TFIIH consists of a number of proteins, some of which have enzymatic activity. In addition to two helicase subunits, TFIIH contains a kinase activity, CDK7, that is able to phosphorylate the CTD of RNA pol II (47). Several studies have demonstrated a direct and specific interaction of Tat with CDK7-containing complexes (36,58,131). Interestingly, all studies that indicate a role for TFIIH in Tat-activated transcription describe an effect on transcription elongation, a step of transcription that has sofar not been associated with TFIIH activity.

The P-TEFb complex was first identified in *Drosophila* nuclear extract and shown to act as a positive transcription elongation factor in *in vitro* transcription assays (117). This multi-subunit complex contains a kinase activity that is able to phosphorylate the RNA pol II CTD in a DRB-sensitive manner. Cloning of the kinase subunit of *Drosophila* P-TEFb revealed homology to human PITALRE, a member of the family of cyclin-dependent kinases (189). Independently, Rice and coworkers identified a Tat-associated kinase (TAK) that controlled Tat-mediated activation of transcription and that was indistinguishable from PITALRE (62). Phosphorylation of the RNA pol II CTD by PITALRE/TAK is also DRB-sensitive. A recent study identified cyclin T as the partner of this cyclin-dependent kinase and PITALRE/TAK was subsequently renamed CDK9 (177). Cyclin T, a component of the P-TEFb complex, was shown to bind Tat directly and to greatly enhance the affinity and specificity of the Tat-TAR interaction.

It was previously recognized that Tat transactivation requires sequences in the loop of TAR that are not recognized by Tat. Combined results from several studies indicated that the loop sequences recognize a cellular factor that is recruited to TAR by Tat (reviewed in (38)). Additional evidence for a loop binding factor that is essential for Tat transactivation comes from studies of the LTR promoter in rodent cell lines. In these cells, transactivation of LTR transcription by Tat is severely impaired, but no longer TAR-loop dependent (9,70). Complementation studies using human-rodent hybrid cells demonstrate that a factor encoded on human chromosome 12 can partially rescue Tat transactivation in rodent cells. This complementation also restored specificity for the loop of TAR (70). The cyclin T component of the P-TEFb complex is a probable candidate for this loop-binding factor, because it is encoded on human chromosome 12 and leads to protection of the TAR loop in RNA probing assays in the

presence of Tat (177). Furthermore, overexpression of human cyclin T in rodent cell lines restored the Tat response.

The combined results from the above mentioned biochemical and genetic studies suggest that Tat functions at a post-initiation step of transcription. It remains to be determined which cyclin-CDK complex is functional *in vivo*. It remains possible that TFIIH and P-TEFb are utilized in a sequential manner to promote phosphorylation and hyperphosphorylation of the RNA pol II CTD, respectively (85,90).

### 4.7 HIV-1 transcription *in vivo* requires chromatin remodeling

It is important to note that HIV-1 transcription *in vivo* involves an integrated viral DNA template, while most HIV-1 transcription studies utilize reconstituted *in vitro* transcription reactions or cells that are transiently transfected with episomal plasmid DNA. Recently, more attention has been given to transcription from an integrated HIV-1 provirus that is packaged into chromatin. Nucleosomes are the basic subunits of chromatin and consist of DNA wrapped around a core of histone proteins. Nucleosomes restrict the ability of transcription factors to access the DNA and thereby inhibit transcription. It has been recognized that chromatin remodeling is a likely prerequisite for efficient transcription from a chromosomal template (94). In particular, the integrated HIV-1 LTR promoter seems to require chromatin remodeling, since transcription is almost completely silenced in this context (87,135) when compared to its activity in *in vitro* transcription assays (131).

Chromatin remodeling requires the acetylation of core histones by Histone Acetyl-Transferase (HAT) enzymes (159). Alternatively, inhibitors of histone de-acetylase enzymes can induce general hyper-acetylation of histones and resolve repressive chromatin structure. Such inhibitors have been shown to activate the HIV-1 LTR promoter and induce virus production from transcriptionally silent proviruses, demonstrating the importance of chromatin remodeling for HIV-1 transcription (166).

The HIV-1 LTR promoter is packaged into two nucleosomes, independent of the site of chromosomal integration. In detail, the two nucleosomes flank a nuclease hypersensitive region that includes the HIV-1 TATA box and Sp1/NF-κB binding sites. While the upstream nucleosome is consistently nuclease-resistant, the downstream nucleosome becomes nuclease sensitive upon activation of the promoter by Tat (167). Thus, Tat protein can mediate the transition from repressive to active chromatin structure (48).

Several recent papers demonstrate that Tat associates with a known cellular HAT enzyme, p300/CBP (13,77,118). The interaction was observed *in vitro* and in *in vivo* by means of co-immunoprecipitation assays and required the basic domain of Tat (77,118). Functional assays with reporter constructs demonstrate a synergistic increase in HIV-1 promoter activity upon co-expression of Tat and p300 (13,77,118). These findings may shed new light on the long-standing notion that HIV-1 Tat affects transcription initiation in addition to increasing the processivity of RNA pol II (118).

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# Chapter 2

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# Optimal Tat-mediated activation of the HIV-1 LTR promoter requires a full-length TAR RNA hairpin

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## ABSTRACT

**HIV-1 transcription from the LTR promoter is activated by the viral Tat protein through interaction with the nascent TAR RNA hairpin structure. The mechanism of Tat-mediated transcriptional activation has been extensively investigated with LTR–CAT reporter genes in transient transfections and, more recently, in infection experiments with mutant HIV-1 variants. Several discrepancies between these two assay systems have been reported. For instance, whereas opening of the lower part of the TAR RNA stem does not affect the promoter activity of an LTR–CAT plasmid in transient assays, the corresponding virus mutant is fully replication-impaired. With the aim to resolve this controversy, we have examined the activity of a set of TAR RNA mutants in transient transfection experiments with a variety of cell types. We now demonstrate that truncated TAR motifs exhibit a severe, but cell-type dependent transcription defect. Whereas full LTR activity is measured in COS cells that have been used regularly in previous transfection assays, a severe defect is apparent in a variety of human cell lines, including T cell lines that are typically used in HIV-1 replication studies. These results suggest the presence of a human protein that participates in Tat-mediated transcriptional activation through binding to the lower part of the TAR stem. Several candidate co-factors have been reported in literature. This study resolves the discrepancy between transfection and infection studies on the requirements of the lower TAR stem structure. The evidence also implies that LTR transcription studies should be performed preferentially in human cell types.**

## INTRODUCTION

The pathogenic human immunodeficiency virus type 1 (HIV-1) encodes not only the three structural genes (*gag*, *pol* and *env*) common to all retroviruses but also six unique gene products, including the Tat protein (1). This protein potently activates transcription from the viral long terminal repeat (LTR) promoter. Tat gains access to the DNA promoter region by binding to the *trans*-acting-responsive RNA element (TAR) that folds into a stable stem–loop structure as part of the nascent transcript. The HIV-1 Tat–TAR axis has been one of the most intensively investigated viral regulatory mechanisms (see reviews, 2,3). Extensive mutational analyses have identified the active domains in both the Tat protein and the TAR RNA element. The initial mutagenesis studies on the TAR RNA motif were done primarily in transiently transfected cells containing a Tat expression plasmid and a second plasmid encoding a reporter gene under transcriptional control of the HIV-1 LTR promoter (4–9). Furthermore, appropriate cell-free assay systems have been developed to study the Tat–TAR interaction and the mechanism of transcriptional activation (10–17). More recently, replication studies with mutant HIV-1 variants demonstrated that both Tat protein (18–22) and its TAR RNA target sequence (23–27) are essential for viral replication, although some level of TAR-independent replication has been reported in activated T lymphocytes (28) and in astrocytic glial cells (29).

Several transient transfection studies suggested that the integrity of the TAR stem, in particular of the upper domain including the single-stranded bulge and loop elements, is important for efficient transcriptional activation by Tat (8,9,30). The identity of the base pairs surrounding the tri-nucleotide bulge was found to be critical both for efficient *trans*-activation (31) and high-affinity binding of the Tat protein (32). In comparison, no or relatively moderate defects were scored for mutations in the lower stem domain of TAR (5,31). For instance, the detailed mutational analysis by Jakobovits *et al.* (5) in human epithelial 293 cells reported 34–39% LTR activity for TAR variants with a triple base substitution in the +7/+18 region of the stem. This defect is relatively minor compared with triple nucleotide substitutions in either the single-stranded bulge or loop element [6 and 2% LTR activity, respectively (5)].

We reported previously a discrepancy between transient transfection assays and virus infectivity studies with TAR-mutated HIV-1 LTR promoter motifs (23). Specifically, we found that a mutant HIV-1 virus with base substitutions in the lower TAR stem is replication-incompetent, although the same TAR mutant is fully transcriptionally active in transient transfections with an LTR–CAT (chloramphenicol acetyltransferase) reporter construct (31). These combined results may suggest an additional role of the TAR hairpin structure in the virus replication cycle, but the observed difference in TAR requirement may also reflect a variation in transcription in these two experimental settings. For

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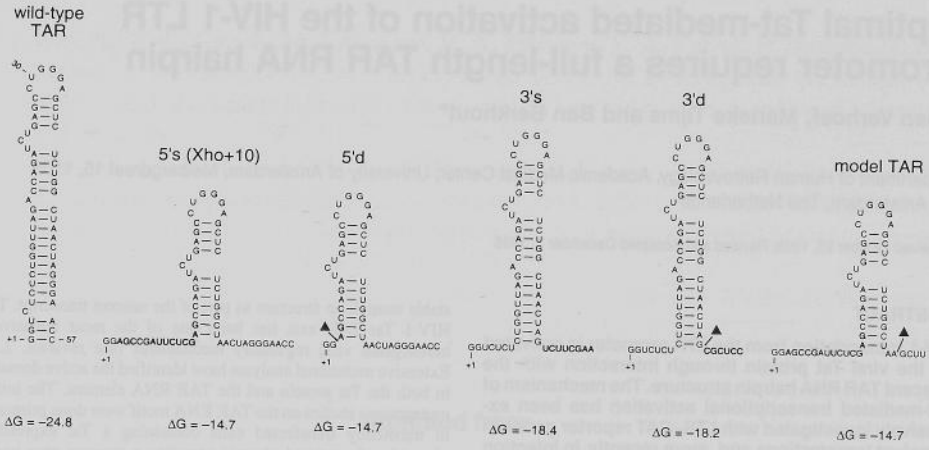


**Figure 1.** Nucleotide sequence and RNA secondary structures of the wild-type and mutant TAR constructs. The HIV-1 TAR hairpin was mutated in the 5′/3′ segment of the lower stem in variants 5′s (Xho+10), 5′d, 3′s, 3′d and model TAR. Substitutions are marked by a grey box, ▲ represents a deletion. The Xho+10/5′s mutation was tested previously in the context of the replicating virus. Nucleotide numbers refer to the position of the 5′TAR element on the genomic RNA, with +1 being the capped G residue. The RNA secondary structures were predicted by free energy minimization with the Zuker algorithm (62), the free energy ΔG (in kcal/mol) was calculated including the terminal stacks. The wild-type TAR stem consists of 23 bp, which is reduced to 11 bp in mutants Xho+10/5′s, 5′d and model TAR and 17 bp in mutants 3′s and 3′d.

instance, the difference may reflect transcription from an integrated versus an unintegrated LTR promoter in the infected and transfected cells, respectively (33). Furthermore, whereas infections are routinely performed in CD4$^+$ T cells, transfections are frequently performed in the non-T cell line HeLa or the non-human cell line COS. Here, we present evidence that a full-length TAR element is required for efficient LTR-transcription in human cell types, but this effect is less obvious in other cells.

## MATERIALS AND METHODS

### DNA constructs

Details on the LTR–CAT plasmids used in this study can be found in previous publications (31,34); the wild-type LTR–CAT contains the complete HIV-1 LTR U3 region upstream, and the *cat* reporter gene downstream of TAR (cloned in the *Hin*dIII site at position +78). Nucleotide numbers refer to the position on the HIV-1 RNA genome, with +1 being the transcriptional start site. The revertant TAR motifs that restore replication of the Xho+10 mutant virus were described previously (23). The proviral sequences were subcloned into LTR–CAT by exchange of the *Pvu*II–*Hin*dIII fragment (position –22/+76). The Tat-expression plasmid pcDNA3-Tat contains the Tat-Rev genomic segment of the HIV-1 isolate pLAI (35) inserted downstream of the cytomegalovirus immediate early promoter in pcDNA3 (Invitrogen). Details of this vector will be described elsewhere [Verhoef and Berkhout, in preparation, see also (36)].

### Cell culture, DNA transfection and CAT enzyme analysis

The lymphocytic T cell lines (SupT1, A3.01, MT2, C8166) were maintained at 37°C and 5% $CO_2$ in RPMI 1640 medium containing 10% fetal calf serum (FCS). Adherent cell lines (COS, HeLa) were grown in Dulbecco's modified Eagle's medium with 10% FCS. Transient transfections were performed with DEAE–dextran [COS and HeLa cells, see (7)] or by electroporation [all T cell lines, see (37)]. Co-transfection of COS cells (~70% confluency on 60 mm dishes) was performed with 1 μg LTR–CAT and 0.1 μg pcDNA3-Tat. HeLa cells (~70% confluency on 60 mm dishes) were transfected with 1 μg of each plasmid, and all T cell lines ($5 \times 10^6$) were electroporated with 2 μg LTR–CAT and 5 μg pcDNA3-Tat plasmid. We did verify that these Tat levels are within the linear range of LTR activation. To measure basal promoter activity, we used 30 μg LTR–CAT to transfect SupT1 cells. Cell extracts were prepared at day 3 post-transfection and assayed for CAT activity with butyryl-CoA in combination with the phase-extraction method as described (38). CAT activities were quantitated in the linear range of the reaction.

## RESULTS

### Truncated TAR hairpins are transcriptionally defective in human cells

The necessary and sufficient sequences for Tat-mediated *trans*-activation have been reported to map between nucleotides +19 and +42 in the HIV-1 LTR [reviewed in (34)]. This region folds the upper half of the TAR hairpin with the typical 3 nucleotide (nt) bulge and 6 nt loop. This structured RNA motif is generally considered to be recognized as part of the nascent RNA transcript by Tat protein and cellular co-factors in the process of transcriptional activation. The Xho+10 mutant contains a sequence substitution upstream of the minimal TAR domain (Fig. 1, mutated segment at position +3/+16 is boxed), resulting in opening of the lower TAR stem. This mutant was previously

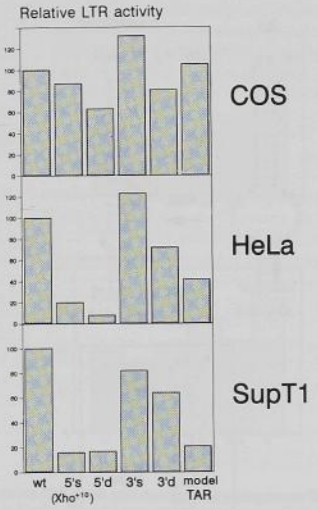


**Figure 2.** Severely truncated TAR elements demonstrate a transcriptional defect in human cells. Three cell types (the COS African green monkey kidney cell line, the HeLa human epithelial, cervix carcinoma cell line and the human SupT1 T cell line) were transfected with the indicated LTR–CAT constructs. The CAT activity measured in transfections of the wild-type LTR–CAT in the presence of Tat was standardized at 100% for each individual cell line. The results shown represent average values of three (COS and SupT1) or four (HeLa) independent DNA transfections, with deviations from the average in the range of 10–20%.

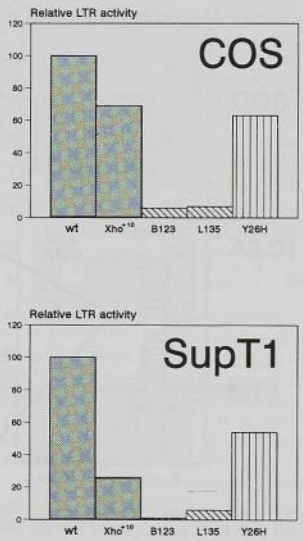


**Figure 3.** The conditional defect of HIV-1 LTR transcription in COS cells is specific for TAR stem mutants. The transcriptional profile of three TAR mutants and one Tat protein mutant was measured in the human T cell line SupT1 and the non-human kidney cell line COS. TAR stem mutant Xho+10 is described in Figure 1. B123 contains a mutated bulge element (UCU to AAG) and L135 a triple substitution in the TAR loop (CUGGGA to AUUGUA); both mutants were described previously (31). The transcriptional activity of these TAR-mutated promoters was measured in combination with the wild-type Tat protein. In addition, we measured the activity of a wild-type LTR promoter in combination with a partially defective Tat mutant containing a single point mutation at amino acid position 26 (Tyrosine to Histidine; mutant Y26H, Verhoef and Berkhout, in preparation). Plotted is the relative LTR activity in the presence of Tat, which is similar to the fold-induction of promoter activity in response to Tat because no differences in basal LTR activity were measured [experiments not shown, see also Fig. 5B and reference (31)]. The CAT activity measured in co-transfections with the wild-type LTR–CAT and Tat constructs was standardized at 100% for the individual cell lines. Experimental variation was found to be <20%.

reported to be fully active in transient COS cell transfections (34), but the same mutation is detrimental to virus replication in human T cells (23). We therefore reexamined the activity of this TAR mutant in a variety of cell types, including monkey kidney COS cells, human epithelial HeLa cells and the human T cells SupT1. LTR–CAT reporter plasmids with the wild-type or Xho+10-mutated TAR element were transfected in the presence of a second plasmid encoding Tat. Cell lysates were prepared 3 days post-transfection and tested for CAT enzyme activity (Fig. 2). Construct Xho+10 retained <20% of the wild-type expression level in both human cell lines HeLa and SupT1. In contrast, >80% transcriptional activity was consistently measured in the monkey COS cells.

To verify this cell type-specific defect in TAR function, we tested a larger set of structurally altered TAR mutants (Fig. 1). Mutant 5′d contains a deletion in the same region that was substituted in mutant Xho+10 (in analogy to the names of the other mutants, we also refer to the Xho+10 mutant as 5′s). Both Xho+10/5′s and 5′d mutants encode an amputated TAR stem (11 bp, $\Delta G = -14.7$ kcal/mol) compared with the wild-type TAR hairpin (23 bp, $\Delta G = -24.8$ kcal/mol). The activity spectrum of 5′d in the three cell lines was indistinguishable from that of Xho+10/5′s; significant activity was scored exclusively in COS cells, and severely reduced expression was measured in HeLa and SupT1 cells (Fig. 2). The 3′s and 3′d mutations affect the 3′ side of the TAR stem, thereby truncating the TAR stem to 17 bp (Fig. 1, $\Delta G = -18.4$ and $-18.2$ kcal/mol, respectively). These plasmids were transcriptionally active in human cells (Fig. 2), indicating that a duplex RNA structure of ~17 bp is sufficient for optimal *trans*-activation in human cells. Another TAR mutant was used previously as model TAR motif in detailed mutational analysis of the upper TAR domain (31). This mutant, model TAR, combines the Xho+10/5′s substitution as in Xho+10 and a deletion on the 3′-side of TAR (Fig. 1; stem consists of 11 bp, $\Delta G = -14.7$ kcal/mol), and a severe transcription defect was scored specifically in the human cell types (Fig. 2). These results suggest that the integrity of the TAR stem is critically important for function in human cells, consistent with the observed replication defect of the corresponding virus mutant in human T cell lines.

### The lower TAR stem, but not the loop or bulge elements, function in a cell type-specific manner

Although many HIV-1 functions have been reported to contribute to viral replication in a cell type-specific manner, such an effect

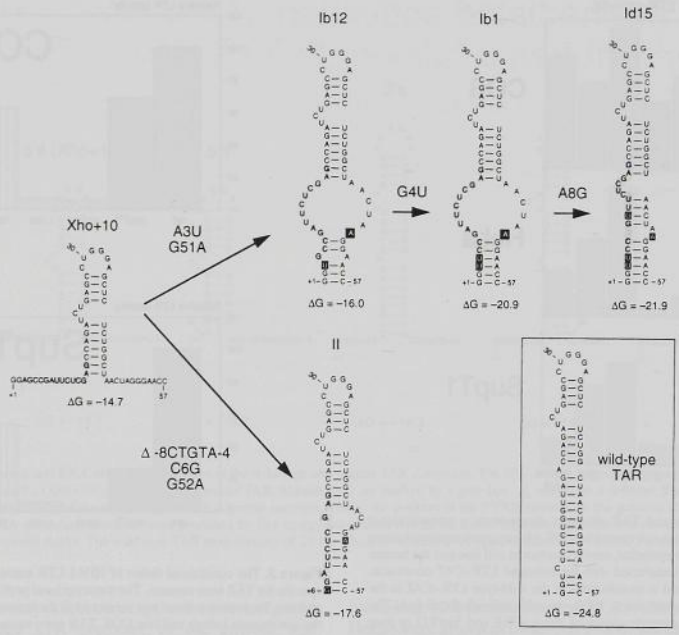


**Figure 4.** Nucleotide sequence and RNA secondary structure of the Xho+10 mutant and revertant TAR hairpins. An HIV-1 variant with the Xho+10 TAR mutation is severely defective in replication, but can give rise to spontaneous revertant viruses. We previously described in detail two reversion experiments [(23); routes I and II, upper and lower part]. The mutated segment in Xho+10 is marked by a grey box. The mutations that became fixated in the revertant genomes are listed next to the arrows of the two evolutionary pathways (e.g. A3U is an A to U transversion at position +3, Δ-8CTGTA-4 represents a 5 nt deletion upstream of the transcriptional start). These acquired mutations are highlighted in the RNA hairpin structures by a black box. These structures were predicted by the Zuker algorithm (62), and the calculated free energies (in kcal/mol, including terminal stacks) are indicated. The number of base pairs in the RNA helixes is as follows; Xho+10 mutant, 11; revertant Ib12, 16, Ib1, 17; Id15, 21; II, 18. The wild-type TAR hairpin (23 bp) is included in the lower right box for comparison.

has not been described for Tat/TAR-mediated LTR transcription. Three explanations can be proposed for this cell-type dependent phenotype. First, it is possible that a cellular factor, involved in Tat-mediated *trans*-activation through binding to the lower TAR stem, is lacking or inactive in COS cells. Second, LTR transcription in COS cells may not require co-factors that bind the lower TAR stem. Third, the mechanism of LTR-transcription is a multistep process and it is possible that the rate of this process is controlled in COS cells at a slow, rate-limiting step that is not apparent in human cells. In all three scenarios, opening of the TAR stem will not influence the transcription rate in COS cells. If a particular rate-limiting step determines the level of LTR transcription in COS cells, other HIV-1 mutants defective in LTR-transcription should also be less overt in this cell type. We therefore tested the cell-type specificity for other TAR mutants and the partially defective Tat protein mutant Y26H. Two additional TAR mutants were tested, either with substitutions in the single-stranded bulge or loop domain (mutants B123 and L135, respectively, see legend to Figure 3 for further details on the substitutions in these mutants). Their transcriptional activity was scored upon transfection of COS and SupT1 cells (Fig. 3). Unlike the Xho+10 stem-mutant that was defective exclusively in SupT1 cells, these TAR mutants were equally defective in the two cell types. Furthermore, the Y26H Tat mutant demonstrated a similarly reduced activity in both cell types (Fig. 3). These results suggest that the cell-type dependent phenotype is rather specific for lower TAR stem mutants, consistent with the first two models.

A previous study reported that the replication defect of TAR-mutated viruses can be complemented by activation of the host T cells by phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin (PHA) (28). Thus, it is possible that the cellular milieu of COS cells is similar to that of PMA/PHA-activated T cells with respect to the function of mutant TAR motifs. To directly test this possibility, we measured the activity of the Xho+10 mutant in the SupT1 T cell line upon activation by PMA, PHA or a combination of both reagents. We measured no increased transcriptional activity in these activated T cells (results not shown).

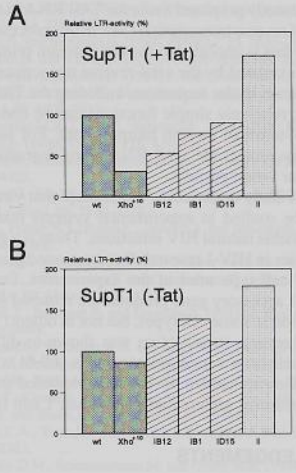


**Figure 5.** Revertant TAR motifs with extended base pairing restore Tat-mediated transcription in T cells. The wild-type, mutant and revertant TAR motifs (see Fig. 4) were transfected with or without wild-type Tat protein in SupT1 T cells (**A**) and (**B**). Transcriptional activity of the wild-type TAR motif was set at 100%. These results represent the average of four (A) and two (B) DNA transfections, with <20% experimental variation.

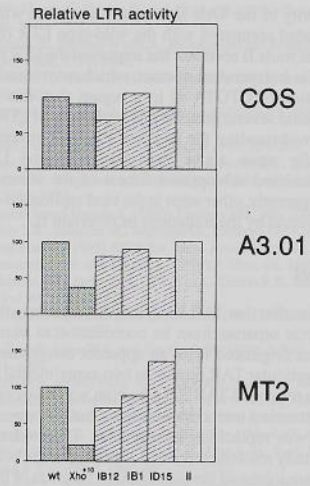


**Figure 6.** Activity spectrum of the TAR mutant-revertants in a variety of cell lines. The A3.01 and MT2 cells are human T cell lines. The CAT activity measured in transfections of the wild-type LTR–CAT in the presence of Tat was standardized at 100% for each individual cell line. The results shown represent average values of two (COS) or three (A3.01 and MT2) independent DNA transfections, with <20% experimental variation.

### Repair of the truncated TAR stem restores transcriptional activity

The analysis so far indicates that an extended RNA hairpin structure is important for optimal TAR function in human cells. However, we cannot exclude alternative mechanistic explanations. For instance, the inactivity of the Xho+10/5′s and 5′d mutants in human cells may reflect a sequence-specific binding of a human co-factor to the +3/+16 TAR sequences, either in the RNA or DNA form. When the Xho+10 mutant virus was used to select for faster replicating revertants, we observed restoration of base pairing of the lower TAR stem by acquisition of additional mutations (23). Several of these TAR revertant structures, obtained in two independent reversion experiments, were now tested in the transient LTR–CAT assay. Three consecutive TAR variants of reversion route I (Fig. 4, upper pathway) and the final structure that evolved in route II (lower pathway) were subcloned into the LTR–CAT plasmid to measure their transcription properties.

In route I, base pairing is gradually restored in the consecutive TAR samples by acquisition of mutations (Fig. 4; Xho+10 mutant, 11 bp; revertants Ib12, 16 bp; Ib1, 17 bp; Id15, 21 bp). Whereas the Xho+10 mutation dramatically reduced the level of Tat-activated LTR transcription in SupT1 cells, the TAR revertants improved the expression level step-by-step and the final ID15 variant produced wild-type levels of CAT enzyme (Fig. 5A). The latter TAR mutant corresponds with a fast replicating revertant virus (23). Thus, the increased LTR expression levels correlated perfectly with the stability and length of the TAR RNA stem. Combined with the results obtained with the initial set of TAR mutants (Fig. 1), these results suggest a correlation between the length of the TAR duplex and its transcriptional activity in human cells.

To further corroborate these results we tested this set of TAR mutant-revertants in two other T cell lines, A3.01 and MT2, and we used COS cells to provide a cellular milieu in which the Xho+10 defect is not manifest. The results of several transfections are summarized in Figure 6 and clearly demonstrate the cell-type specific defect of mutant Xho+10 and the subsequent recovery of transcriptional activity in route I revertants. To test whether the transcriptional defect/repair was specific for Tat-mediated transcription from the LTR promoter, we tested this same set of mutant–revertant TAR motifs in the absence of the Tat *trans*-activator protein. We found that the basal LTR promoter activity was not sensitive either to opening of the TAR stem in Xho+10 or to the subsequent repair of this motif in the three TAR revertants of pathway I (Fig. 5B).

A different TAR repair mechanism is seen in route II (Fig. 4). A 5 nt deletion upstream of the transcription start site (+1) shifts transcription initiation towards the +6 position within TAR (23). This modification apparently removes the 5′ dangling end and subsequent mutation of the new start site results in an abbreviated TAR hairpin with a closed stem. Consistent with the results obtained for the route I revertant, the route II revertant did increase its Tat-mediated transcriptional activity compared with the parental Xho+10 mutant (Fig. 5A). However, the route II revertant is somewhat exceptional in that its transcriptional activity is significantly higher than that of the wild-type LTR promoter. This increased transcription was measured for this TAR II revertant in all cell types tested (Fig. 6). Furthermore, basal

promoter activity of the TAR II revertant measured without Tat was also elevated compared with the wild-type LTR (Fig. 5B). Apparently, the route II revertant has improved the LTR promoter activity in a Tat-independent manner, which may correlate with the 5 nt deletion (-8CTGTA-4) in a region that was reported previously to bind several proteins that repress basal LTR activity (39–41). Notwithstanding the fact that the TAR revertant II is transcriptionally more active than the wild-type LTR, we previously measured sub-optimal fitness of the corresponding virus (23). Apparently, other steps in the viral replication cycle are negatively affected by the mutations in revertant II.

## DISCUSSION

We suggested earlier that TAR RNA may have a role in the HIV-1 replication cycle separate from its contribution to transcription (23). This idea originated from an apparent discrepancy in the activity of a particular TAR mutant in two experimental systems. Specifically, a truncated TAR RNA hairpin was transcriptionally competent in transient transcription assays, but the corresponding virus mutant was replication-incompetent. The evidence presented in this study resolves this issue. Opening of the bottom part of the TAR stem inhibited the transcription function of the HIV-1 LTR promoter in human T cells, the cell type ordinarily used in HIV-replication studies. Previous transfection studies overlooked this transcription defect because it is cell-type dependent and not observed in COS cells. Thus, the integrity of the TAR RNA stem is critical for efficient Tat-mediated LTR transcription and the first definition of the borders of the minimal TAR domain [position +19 to +42, (34)] should be modified accordingly.

The cell-type specific behaviour of the lower TAR stem mutant is rather unique in that other transcriptionally defective TAR mutants display no such cell-type differences. This argues against a general phenomenon, for instance caused by a particular rate-limiting step during LTR-transcription in COS cells. We therefore favour a more direct explanation in which the lower TAR stem contributes in a cell-type specific manner to the transcription process by binding of a co-factor. LTR transcription in COS cells does either not require such a lower TAR stem binding factor, or this co-factor is absent from COS cells. Several TAR RNA-binding factors have been reported (42–55), but only those proteins that require an extended TAR stem for binding can explain the defect of TAR mutants such as Xho+10. The revertant data suggest that binding of this cofactor occurs without strict specificity for the sequence of the lower stem region. We cannot exclude an alternative mechanism, with a COS cell-specific factor that binds to the upper TAR region and that can overcome the requirement for an extended TAR stem.

The present study demonstrates that full-length TAR is required for optimal LTR transcription, thereby explaining the severe replication defect of HIV-1 variants with a truncated TAR stem. However, this does not rule out any additional role for this sequence/structure motif in the viral replication cycle, and several putative functions have been proposed [reviewed in (56)]. In this respect, we note that TAR is part of the repeat (R) region of the LTR that encodes a double hairpin motif [TAR and the 'polyA-hairpin', (57)] that is present at both the extreme 5′ and 3′ ends of all HIV-1 transcripts. These structures may actively participate in one of the many functions of the HIV-1 leader RNA that involve multiple RNA–RNA and RNA–protein contacts, e.g. RNA packaging, dimerization or reverse transcription. Interestingly, a recent study proposed a role for TAR RNA in the process of initiation of reverse transcription (58). In this process, a cellular tRNA primer that is annealed to the downstream primer-binding site (PBS) is extended by the viral reverse transcriptase enzyme over the upstream leader sequences, including the TAR element. Alternatively, relatively simple functions can be considered for the 5′- and 3′-terminal tandem hairpin motif. For instance, the structures may protect the viral RNA from degradation in the infected cell or virion particle.

Finally, this study underscores the concept that viral mechanism should be studied in experimental systems that model as closely as possible natural HIV infections. There is a growing list of controversies in HIV-1 research that are caused primarily by a difference in cell type used in the experiments. For instance, several HIV-1 accessory gene products are required for efficient virus replication in some cell types, but not in others (59) and the efficiency of reverse transcription was shown to differ significantly in T cell lines versus primary cells (60,61). This study reveals that the mechanism of Tat/TAR-mediated transcription should be performed preferentially in human T cell lines.

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# Chapter 3

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# Paracrine Activation of the HIV-1 LTR Promoter by the Viral Tat Protein Is Mechanistically Similar to *Trans*-Activation within a Cell

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The HIV-1 Tat protein activates transcription of the viral LTR promoter through interaction with the nuclear transcription machinery of the host cell. Tat can also activate the LTR promoter in a paracrine or *inter*-cellular manner by a yet unknown mechanism. One possibility is that Tat protein itself is secreted by cells and taken up by other cells. According to this mechanism, *inter*-cellular transcriptional activation by Tat should be very similar to *intra*-cellular *trans*-activation in Tat-producing cells. A large number of cytokine genes was recently reported to be Tat-responsive, raising the possibility that such cytokines and the corresponding cellular transduction pathways are involved in *inter*-cellular Tat action. The transcriptional events in such an indirect route are likely to differ from *intra*-cellular Tat action. To discriminate between a direct or indirect mechanism of *inter*-cellular Tat action, we compared the activity of a set of Tat mutants and different promoter constructs in *inter*-cellular and *intra*-cellular transcriptional activation. Identical results were obtained in both assays, suggesting that Tat protein itself is exported by one and transported into the nucleus of another cell. The demonstration that Tat antibodies specifically inhibit the *inter*-cellular route is also consistent with cell-to-cell transport of the Tat protein. Furthermore, we found that the second Tat coding exon, including the RGD motif that has been proposed to interact with an integrin receptor, is not required for cellular uptake of the Tat protein. 

## INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) Tat protein is a potent activator of viral gene expression and replication (Luciw, 1996). Tat is a nuclear protein (Hauber *et al.*, 1989; Ruben *et al.*, 1989; Hauber *et al.*, 1987; Chin *et al.*, 1991) that is brought in proximity to the transcription machinery because of its ability to bind to the TAR RNA hairpin structure as part of the nascent transcript (Berkhout *et al.*, 1989; Dingwall *et al.*, 1989). Mutational analyses have shown that Tat-activation requires not only the trinucleotide bulge that forms the actual Tat binding site, but also the apical loop and the stem domain of TAR RNA (Feng and Holland, 1988; Cordingley *et al.*, 1990; Dingwall *et al.*, 1990; Roy *et al.*, 1990; Weeks and Crothers, 1991; Garcia *et al.*, 1989; Berkhout and Jeang, 1989; Berkhout and Jeang, 1991; Klaver and Berkhout, 1994a). The molecular mechanism by which Tat activates HIV-1 gene expression has been the subject of intense investigation (Cullen, 1993). Tat activates transcription of the long terminal repeat (LTR) promoter by increasing the efficiency of transcriptional initiation as well as increasing the efficiency of transcriptional elongation. Numerous cellular factors were proposed to be involved in Tat-mediated activation of transcription. These proteins bind either to Tat itself or to the TAR RNA structure. Candidate Tat-binding factors include a cellular protein kinase (Herrmann and Rice, 1993, 1995), a factor with Tat-complementing activity in cell-free transcription assays (Zhou and Sharp, 1995) and other proteins (Fridell *et al.*, 1995). TAR-binding proteins include the loop-specific protein TRP-185, RNA polymerase II, and other putative cofactors (Wu *et al.*, 1991; Sheline *et al.*, 1991; Gatignol *et al.*, 1991; Wu-Baer *et al.*, 1996). In addition, the Tat-response is likely to entail several protein–protein contacts between the Tat- and TAR-associated factors and the general DNA-binding transcription factors and transcriptional activators, including NF-κB, Sp1, TBP, and LBP-1 (Kashanchi *et al.*, 1994; Wu-Baer *et al.*, 1995; Veschambre *et al.*, 1995; Jeang *et al.*, 1993; Keen *et al.*, 1996; Mavankal *et al.*, 1996).

Several assays were developed to study the mechanism of Tat-mediated transcriptional activation. The standard Tat assay involves cotransfection of a Tat-expression vector and an LTR-CAT reporter construct. Protein transfection methods such as "scrape-loading" (Frankel and Pabo, 1988; Gentz *et al.*, 1989) and electroporation (Kashanchi *et al.*, 1992; Verhoef *et al.*, 1993) have also been developed to introduce Tat protein into tissue culture cells. It is believed that these procedures transiently damage cell membranes, allowing molecules in the medium to enter the cytoplasm. Surprisingly, it was also found that Tat, either the recombinant form produced in *Escherichia coli* or a synthetic peptide, can be taken up by cells *in vitro*. The current idea is that Tat subsequently enters the nucleus, leading to *trans*-activation of the HIV-

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1 LTR promoter (Green and Loewenstein, 1988; Frankel and Pabo, 1988; Mann and Frankel, 1991; Frankel *et al.*, 1989). Furthermore, Tat is released into the extracellular medium during acute infection of T cells by HIV-1 or after transfection of a Tat expression plasmid (Ensoli *et al.*, 1993). These combined results suggest that transcriptionally active Tat protein can be secreted from one and taken up by a second cell ("*inter*-cellular" *trans*-activation), although little detail on the molecular mechanisms of Tat protein secretion and uptake is currently available.

HIV-infected T cells and Tat plasmid-transfected COS cells were found to secrete substantial quantities of Tat protein by an unknown mechanism (Ensoli *et al.*, 1993). The absence of a recognizable signal sequence in Tat does not rule out protein secretion because a number of growth factors lack such signals (Acland *et al.*, 1990). Cellular uptake of Tat was suggested to occur through binding of Tat to the cell surface via specific (receptor-mediated) interactions. In particular, the C-terminal Tat domain encoded by the second coding exon ("exon 2") contains the arginine–glycine–aspartate sequence (the RGD motif) that is present in extracellular matrix proteins and responsible for cell adhesion through binding to integrin receptors (Brake *et al.*, 1990a; Barillari *et al.*, 1993). The *in vivo* biological relevance of these *inter*-cellular effects of Tat is yet unknown, but there may be situations where extracellular Tat reaches sufficient concentrations to influence transcription in other cells. For instance, extracellular Tat may activate HIV-1 gene expression in cells infected with a Tat-defective provirus. Paracrine Tat may also affect the physiology of uninfected cells by deregulation of cellular gene expression (see below).

Transcriptional activation by Tat was initially believed to be highly specific for the HIV-1 LTR promoter because of strict requirements for both the unique TAR RNA attachment site in the nascent transcript (Berkhout *et al.*, 1989) and a specific set of upstream promoter-enhancer motifs (Berkhout *et al.*, 1990). However, Tat can *trans*-activate the LTR in a TAR-independent manner under certain circumstances (Harrich *et al.*, 1990; Taylor *et al.*, 1992) and Tat can also modulate the expression of a variety of cellular genes (Chang *et al.*, 1995). For instance, Tat stimulates immunoregulatory cytokine genes including tumor necrosis factors $\alpha$ and $\beta$ (TNF$\alpha$ and TNF$\beta$) (Buonaguro *et al.*, 1992, 1994; Sastry *et al.*, 1990), transforming growth factor $\alpha$1 and $\beta$1 (TGF$\alpha$1 and TGF$\beta$1 (Cupp *et al.*, 1993; Lotz *et al.*, 1994; Nabell *et al.*, 1994; Zauli *et al.*, 1993b), the interleukins IL-2 and IL-6 (Purvis *et al.*, 1992; Hofman *et al.*, 1993; Iwamoto *et al.*, 1994; Scala *et al.*, 1994; Zauli *et al.*, ,1993a), and Tat activates several cytokine receptors including IL-2R$\alpha$ and IL-4R (Purvis *et al.*, 1992). Tat also decreases transcription of cellular genes. For instance, down-regulation of the major histocompatibility complex class I gene (MHCI) has been reported (Howcroft *et al.*, 1993), which may provide a mechanism for virus escape from the host immune response. These Tat effects were observed primarily in tissue culture experiments, but similar results were recently reported in transgenic mice (Brady *et al.*, 1995). Deregulation of cellular genes by Tat may explain the observation that numerous cytokine levels are elevated in the serum of HIV-1-infected individuals (reviewed in (Chang *et al.*, 1995)). In this study, we tested whether soluble cellular factors and/or cytokines are involved in *inter*-cellular LTR-activation. The results indicate that Tat protein itself is exported and taken up by cells.

## MATERIALS AND METHODS

### Cell lines and plasmids

The lymphocytic T cell line SupT1 was maintained at 37° and 5% $CO_2$ in RPMI 1640 medium containing 10% fetal calf serum (FCS). COS cells were grown in Dulbecco's modified Eagle's medium with 10% FCS. The LTR-CAT reporter construct was described previously (3'LTR-CAT in (Klaver and Berkhout, 1994b). The Tat-expression plasmid pcDNA3-Tat contains the Tat-Rev genomic segment of the HIV-1 isolate pLAI (Peden *et al.*, 1991) inserted downstream of the cytomegalovirus immediate early promoter in pcDNA3 (Invitrogen). Details of this vector and the mutagenesis protocol will be described elsewhere (Verhoef and Berkhout, in preparation). The SV40-CAT and CMV-CAT plasmids were described previously (Koken *et al.*, 1994). The RSV-Luc plasmid contains the Luciferase reporter gene under transcriptional control of the Rous sarcoma virus LTR promoter and was kindly provided by Dr. Hans Pannekoek (Department of Biochemistry, University of Amsterdam). Mutants of the HIV-1 LTR-CAT construct with specific base substitutions in the TAR bulge and loop domains were described previously (Berkhout and Jeang, 1991).

### Transfections

Transient transfections were performed with DEAE-dextran (COS cells; see (Berkhout and Jeang, 1989)) or by means of electroporation (SupT1 T cells, see (Das *et al.*, 1995)). In a typical *inter*-cellular Tat assay, COS cells (80% confluency on 60-mm dishes) were transfected with 10 $\mu$g pcDNA3-Tat plasmid (wild-type or mutant) and SupT1 cells ($5 \times 10^6$) with 10 $\mu$g LTR-CAT. The transfected cells were cultured in RPMI medium with 10% FCS for 24 hr. After being washed, the T cells were resuspended and added to the dish with COS cells in a total volume of 5 ml. Fresh medium (2 ml) was added 48 hr after transfection and the SupT1 cells in the supernatant were harvested at 72 hr posttransfection. Cell extracts were prepared and assayed for CAT activity as described (Seed and Sheen, 1988). CAT activities were quantitated in the linear range of acetylation. For the Luciferase assay, SupT1 T cells were collected by centrifugation

and resuspended in 50 $\mu$l 100 m*M* K-phosphate buffer (pH 7.8), and then DTT was added to 5 m*M*. Cells were lysed by three cycles of freeze-thawing (1 min −80° in $CO_2$/ethanol and 2 min 37°), and the extract was cleared by centrifugation. Extract (20 $\mu$l) was mixed with 280 $\mu$l buffer (25 m*M* glycylglycine, pH 7.8, 15 m*M* MgSO4, 3.3 m*M* ATP, 100 $\mu$g/ml BSA) and the reaction was initiated by addition of 100 $\mu$l luciferin (Sigma). Light emission was measured for 10 sec in a luminometer.

For cocultivation in the absence of COS-SupT1 cell–cell contact, the COS cells were seeded and transfected with 5 $\mu$g pcDNA3-Tat in 35-mm Transwell plates (Costar). At the same time, 2.5 × $10^6$ SupT1 cells were transfected with 5 $\mu$g LTR-CAT. At Day 1 posttransfection, the T cells in 2.5 ml RPMI were added to the upper compartment of the Transwell plate. Fresh medium (1 ml) was added at 48 hr posttransfection and the SupT1 cells were harvested after 72 hr. In other experiments, cocultivation was performed in the presence of Tat-specific antibodies. Subconfluent COS cells in 35-mm dishes were transfected with 5 $\mu$g pcDNA3-Tat, whereas several independent SupT1 transfections (10 $\mu$g LTR-CAT/5 × $10^6$ cells) were pooled after 24 hr and split such that each COS dish received 2.5 × $10^6$ transfected SupT1 cells in 2.5 ml RPMI. The transfected SupT1 cells were first mixed with the antibody solution, and subsequently added to the Tat-producing COS cells. Regular cotransfections (*intra*-cellular assay) were performed in SupT1 cells with 1 $\mu$g pcDNA3-Tat and 2 $\mu$g LTR-CAT.

### Western blotting

COS cells (80% confluency, 90-mm dish) were transfected with 10 $\mu$g of the indicated pcDNA3-Tat constructs. Cells were washed in phosphate-buffered saline (PBS) after 3 days and resuspended in 1 ml 2× SDS sample buffer. Proteins were resolved on a 20% SDS–polyacrylamide gel and transferred to Immobilon-P (Millipore) at 60 V for 16 hr. The membrane was subsequently blocked with 5% nonfat dry milk in PBS buffer with 0.05% Tween-20, incubated with Tat monoclonal antibodies (diluted 1 in 1000 in PBS), kindly provided by Dr. C. Debouck (Brake *et al.*, 1990b), and developed with the BCIP-NBT protocol as described (Das *et al.*, 1995) (Table 1).

## RESULTS

### Several hypothetical routes for *inter*-cellular Tat action

In contrast to the regular Tat-assay that occurs within one cell ("*intra*-cellular"), we will refer to the two-cell Tat mechanism as the "*inter*-cellular" Tat assay (Fig. 1). The Tat-transfected cell that expresses Tat protein is the "donor cell" and the LTR-CAT-transfected cell is the "acceptor cell". The mechanism by which Tat modulates gene expression

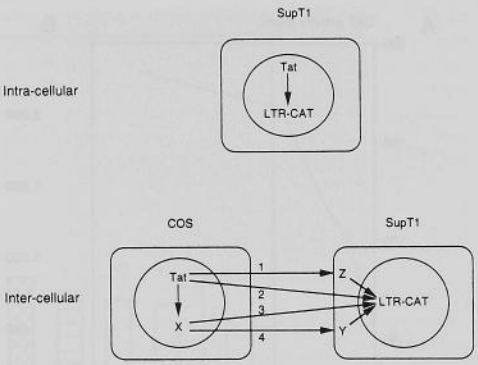



FIG. 1. Schematic of four potential routes for *inter*-cellular Tat-mediated activation of the viral LTR promoter. The top panel shows *intra*-cellular activation of the HIV-1 LTR promoter by Tat protein produced in a cotransfected SupT1 cell. The bottom panel shows the Tat-producing COS cell (donor cell) that is cocultured with an LTR-CAT-transfected SupT1 cell (acceptor cell). Tat is secreted by COS cells in routes 1 and 2, followed either by uptake in SupT1 cells and nuclear transport (the direct route 2) or activation of a putative cellular cofactor Z (route 1, see text for details). According to the hypothetical routes 3 and 4, Tat activates the expression of a secreted cofactor X in COS cells. This cytokine-like factor subsequently activates transcription in SupT1 cells through direct or indirect pathways (routes 3 and 4, respectively, with involvement of cofactor Y in the latter scenario).

in the *inter*-cellular route is generally considered to be direct with cellular secretion and uptake of Tat protein, which is supposed to interact with cellular transcription factors in the nucleus of the acceptor cell (route 2 in the schematic of Fig. 1). However, indirect routes involving cellular factors either in the donor or acceptor cell can be envisaged. First, secreted Tat may activate a signal transduction pathway in the acceptor cell by interaction with a cell surface receptor or, upon entering of the cell, by interaction with a cytosolic protein (putative cofactor Z in route 1). A putative target for Tat are the integrin receptors, signalling molecules that transmit information from the extracellular domain to the cytoplasmic domain via a number of signalling pathways including PKC, MAP kinases, changes in intracellular pH, calcium concentration, and membrane potential (Schwartz *et al.*, 1995). Alternatively, Tat may enter the acceptor cell and interact with a regulatory cofactor, e.g., the Tat-associated protein kinase (Herrmann and Rice, 1995). Second, because Tat induces a large number of cellular proteins with a paracrine function in the producer cell (see Introduction), these cellular proteins may play a role in the *inter*-cellular mechanism. The Tat-induced cytokines may affect gene expression in the acceptor cell through direct or indirect pathways (routes 3 and 4, with a Tat-induced cytokine X and a cofactor Y). For instance, the Tat-induced cytokine TNF can activate the transcription factor NF-$\kappa$B in the acceptor cell (Westendorp *et al.*, 1995).

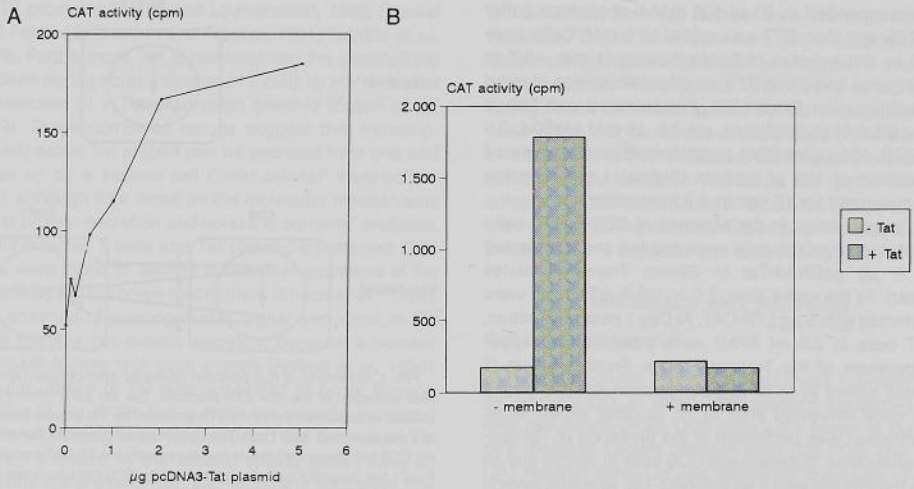



FIG. 2. *Inter*-cellular Tat activity requires direct cell–cell contact. (A) Dose–response curve of LTR-CAT activation in SupT1 cells upon coculture with COS cells transfected with increasing amounts of the Tat plasmid. (B) Culture of SupT1-LTR-CAT cells with mock-transfected (– Tat) or Tat-transfected COS cells. The two cell types were cocultured in the presence or absence of a 0.45-µm filter (+/– membrane).

### The activity of Tat mutants is similar in *intra*- versus *inter*-cellular LTR-activation

The *inter*-cellular Tat assay involves the coculture of Tat-producing cells and LTR-CAT-containing cells. This assay is schematically depicted in Fig. 1 and was performed essentially as described in previous studies (Helland *et al.*, 1991; Marcuzzi *et al.*, 1992a; Marcuzzi *et al.*, 1992b). In brief, adherent COS cells were transfected transiently with the pcDNA3-Tat plasmid and nonadherent SupT1 cells were transfected with the LTR-CAT reporter. When SupT1 reporter cells were cocultivated for 48 hr with the COS-Tat cells, a 4- to 20-fold increase in CAT activity was consistently observed (e.g., Figs. 2A and 2B). Titration of the pcDNA3-Tat plasmid demonstrates a linear response of LTR-induced transcription in the acceptor SupT1 cells (Fig. 2A). Cocultivation with a membrane that prevents cell-to-cell contact abolished *inter*-cellular Tat activation (Fig. 2B). This result is consistent with published evidence (Helland *et al.*, 1991) and indicates a strict requirement for cell-to-cell contact, ruling out the involvement of a soluble cytokine.

To study the mechanism of Tat-mediated *trans*-activation of the LTR promoter in the coculture, we compared the activity of a set of Tat mutants in the *intra*- versus *inter*-cellular assay. We reasoned that if Tat itself is secreted and taken up by cells (route 2), Tat mutants should activate the LTR-promoter with approximate equal efficiency in the two assay systems. On the other hand, if Tat exerts its *inter*-cellular effect through cofactors that are not involved in *intra*-cellular *trans*-activation, some Tat mutants are expected to differ in their transcriptional activation capacity in the two assay systems. It is important to include partially defective Tat mutants in this comparative analysis. Many of the previously described Tat mutants are completely inactive (Jeang, 1995) and the potential problem with such mutants is that their conformation may be affected so dramatically that they will be fully inactive in all assays. Partially defective Tat mutants will be more informative in this comparative analysis.

Four conserved aromatic amino acids within the Cysteine-rich and core domains of the HIV-1 Tat protein were individually replaced by aromatic or nonaromatic amino acids. The mutant proteins were named by appending the amino acid present in wild-type Tat, the residue number, and the amino acid present in the mutant at this position. The LTR-*trans*-activation capacity of these mutants in a standard *intra*-cellular assay in SupT1 cells is presented in Fig. 3A. A complete spectrum of activities was obtained with this set of Tat mutants. For instance, replacements at amino acid position 26 that conserved the aromatic nature of this residue had little effect on the Tat activity, but substitution for Alanine (Y26A) reduced the activity to less than 10%. In contrast, any mutation of the Tyrosine residue at position 47 does significantly reduce the activity to 10–40% of the wild-type Tat protein. Next, we used this collection of Tat mutants in the *inter*-cellular assay where LTR activation is scored in SupT1 cells, but Tat is produced by COS cells (Fig. 3B). Very similar activities were measured for the Tat mutants in this assay compared with the *intra*-cellular assay. This

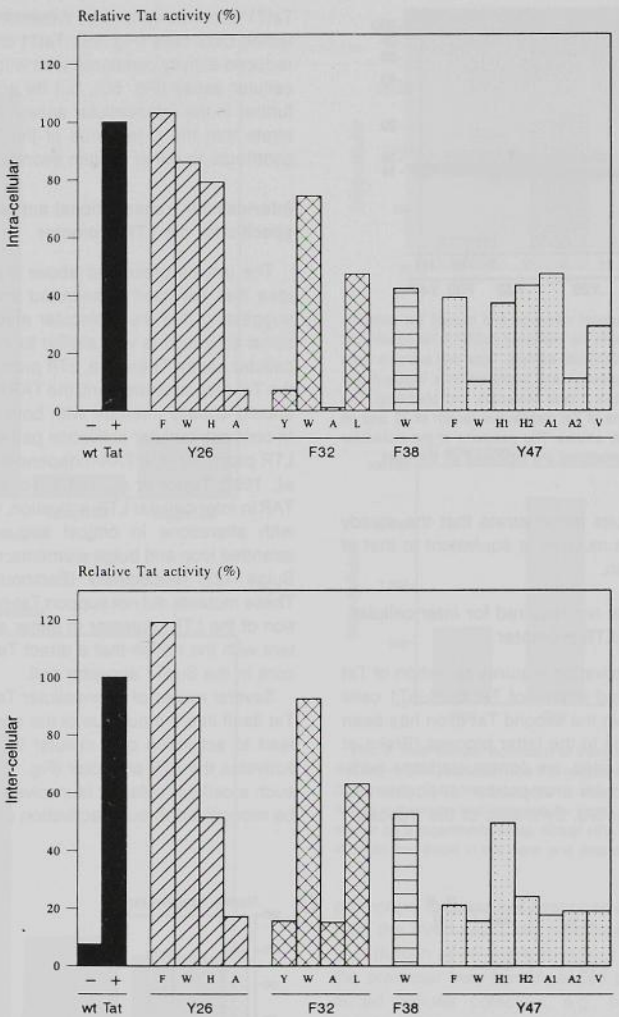



FIG. 3. Comparison of the activity spectrum of a set of Tat mutants in the *intra*-cellular (Top) versus *inter*-cellular assay (Bottom). LTR activity in the absence and presence of wild-type Tat (–/+ wt Tat) is represented by filled bars. The relative promoter activity obtained in the presence of wt Tat was set at 100%. Activities are means of 3 separate experiments, with less than 25% variation in values between experiments. In a typical assay, absolute acetylation levels (after subtracting for background counts) were approximately 20,000 and 2,000 cpm for the *intra*- and *inter*-cellular assay, respectively. The Tat mutants are termed according to the position and type of amino acid substitution. The 1-letter amino acid code was used: Y, tyrosine; F, phenylalanine; W, tryptophan; H, histidine; A, alanine; L, leucine; V, valine. The mutant pairs Y47H1/Y47H2 and Y47A1/Y47A2 encode a similar amino acid (histidine and alanine, respectively), but use different codons.

result is consistent with a direct mechanism of action in which transcriptionally active Tat is exported by COS cells and taken up into the nucleus of SupT1 T cells (route 2 in Fig. 1).

Amino acid substitutions may affect the stability of the Tat mutants and reduced steady state levels of Tat in the COS donor cells will equally affect the direct and indirect routes of *inter*-cellular Tat action. In order to rule out this possibility, we performed Western blot analysis on some of the inactive Tat variants produced in COS

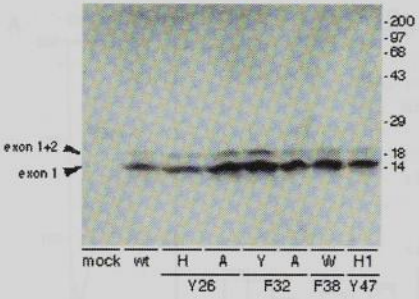



FIG. 4. Western blot analysis of wild-type and mutant Tat proteins. COS cells were transfected with the indicated pcDNA3-Tat constructs. mock is a mock-transfected COS cell sample. Total cell extracts were prepared at 2 days posttransfection and analyzed on a Western blot that was stained with Tat MAb 2 (see Materials and Methods). The exon 1 and exon 1 + 2 forms of Tat (respective length of 72 and 86 amino acids) are indicated by arrows. The positions of the molecular mass marker proteins (in kilodaltons) are indicated on the right.

cells (Fig. 4). The results demonstrate that the steady state level of these Tat mutants is equivalent to that of the wild-type Tat protein.

### The second Tat exon is not required for *inter*-cellular *trans*-activation of the LTR promoter

*Inter*-cellular *trans*-activation requires secretion of Tat protein by COS cells and uptake of Tat by SupT1 cells and the RGD motif within the second Tat exon has been suggested to play a role in the latter process (Brake *et al.*, 1990a). To test this idea, we constructed one additional *tat* gene mutant with a stopcodon at position 72 at the exon-border (Fig. 5A). Synthesis of the truncated Tat71 form was verified by Western blot analysis of transfected COS cells (Fig. 5B). Tat71 displayed a somewhat reduced activity compared with wild-type Tat in the *intra*-cellular assay (Fig. 5C), but its activity did not decline further in the *inter*-cellular assay. These results demonstrate that the C-terminus of the Tat protein does not contribute to either protein secretion or protein uptake.

### *Inter*-cellular transcriptional activation by Tat is specific for the LTR promoter

The results presented above are consistent with the idea that Tat itself is secreted and taken up by cells, suggesting that the molecular mechanism of transcriptional activation is very similar for the *intra*- versus *inter*-cellular assay. Therefore, LTR promoter mutations within the Tat-response element, the TAR RNA hairpin structure, should equally interfere with both transcription assays. In contrast, cellular activation pathways can activate the LTR promoter in a TAR-independent manner (Harrich *et al.*, 1990; Taylor *et al.*, 1992). To test the importance of TAR in *inter*-cellular LTR-activation, we used TAR variants with alterations in critical sequences of the single-stranded loop and bulge elements, mutants Loop135 and Bulge 123, respectively (Berkhout and Jeang, 1991). These mutants did not support Tat-mediated *trans*-activation of the LTR promoter in either assay (Fig. 6), consistent with the notion that a direct Tat-TAR interaction occurs in the SupT1 acceptor cell.

Several routes of *inter*-cellular Tat action do not bring Tat itself into the nucleus of the acceptor cell, but rather lead to activation of a cellular transcription factor that activates the LTR promoter (Fig. 1, routes 1, 3, and 4). If such a cellular cofactor is involved, its role can perhaps be recognized through activation of other transcriptional

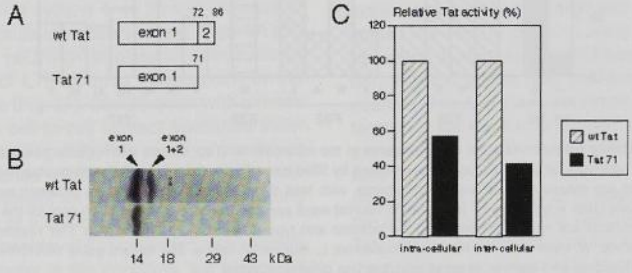
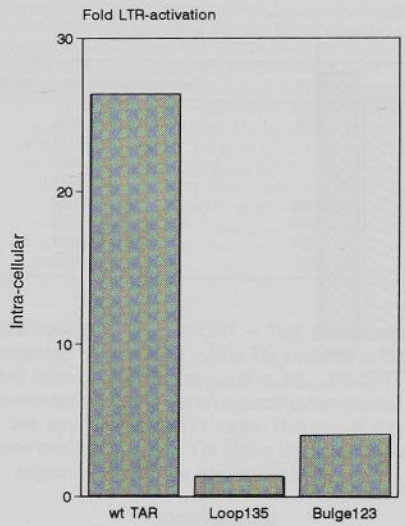



FIG. 5. The second Tat exon is not required for *inter*-cellular activity. (A) Schematic representation of full-length Tat and the truncated exon-1 form. A stopcodon was introduced at codon position 72 in the Tat71 mutant. (B) Western blot analysis of Tat protein expression in COS cells transfected transiently with wt Tat and the Tat71 mutant plasmid. The blot was stained with MAb 4. Wild-type Tat is expressed as the 1-exon and the 1 + 2 exon form, which are 72 and 86 amino acids in length, respectively. The positions of the molecular mass marker proteins (in kilodaltons) are indicated. (C) Comparison of the relative activity of wt and truncated Tat71 in the *intra*- and *inter*-cellular assay. Activities are means of 3 separate experiments, with less than 25% variation in values between experiments. The activity of wt Tat was set at 100% for each assay (see legend to Fig. 3 for details).



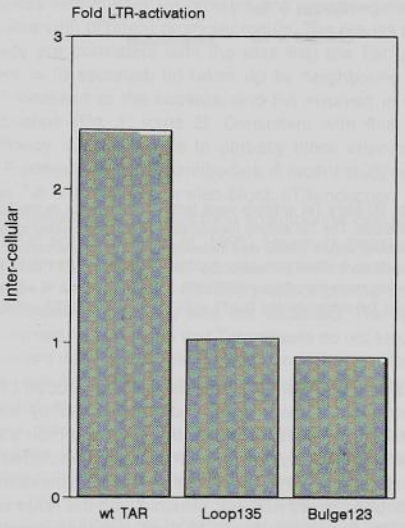




FIG. 6. *Inter*-cellular activation of the HIV-1 LTR promoter by Tat is dependent on the TAR RNA motif. Tat-mediated activation of the wild-type and TAR-mutated HIV-1 LTRs was assayed *intra*- and *inter*-cellularly. The Loop135 mutant contains a triple substitution in the TAR loop (CUGGGA to AUUGUA) and Bulge123 contains a mutated bulge element (UCU to AAG). Plotted is the fold-induction of LTR activity in response to Tat, no differences in basal LTR promoter activity was measured.

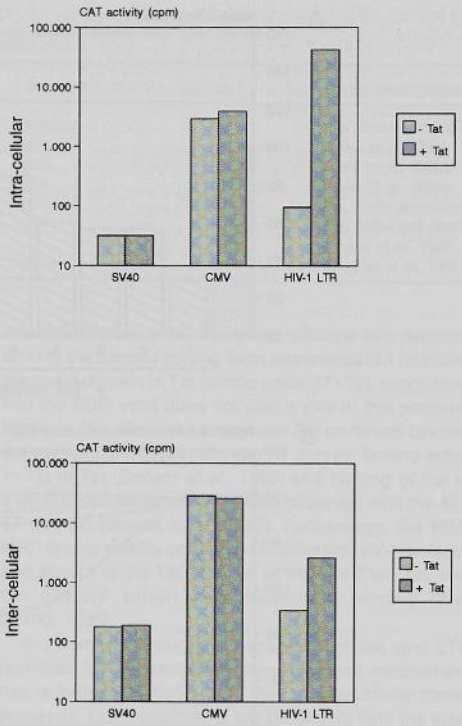



FIG. 7. *Inter*-cellular activation of transcription by Tat is specific for the HIV-1 LTR promoter. The Tat-responsiveness of three promoters (SV40 early, CMV immediate early, and the cognate HIV-1 LTR) was tested in the *intra*- and *inter*-cellular assay. Absolute CAT activities are shown on a logarithmic scale. Actual HIV-1 LTR-induction levels were 450-fold and 8-fold in the *intra*- and *inter*-cellular assay, respectively.

promoters that are not responsive to Tat. For instance, both the SV40 and CMV promoters are not Tat-responsive (Koken *et al.*, 1994; Berkhout and Jeang, 1992), but the promoter may be influenced by the putative Tat-induced cellular cofactors, e.g., both these promoters should respond to activation of the NF-κB protein (Westendorp *et al.*, 1995). Both the weak SV40 and the strong CMV promoter were not affected by Tat expression in cocultured cells (Fig. 7, *inter*-cellular assay), which is the expected condition if Tat itself exerts the *inter*-cellular effects. Similar results were obtained with the Rous sarcoma virus LTR promoter (experiments not shown, see also Fig. 8).

### Inhibition of *inter*-cellular *trans*-activation by Tat antibodies

Antibody-blocking experiments can provide direct evidence for *inter*-cellular transport of the Tat protein, but

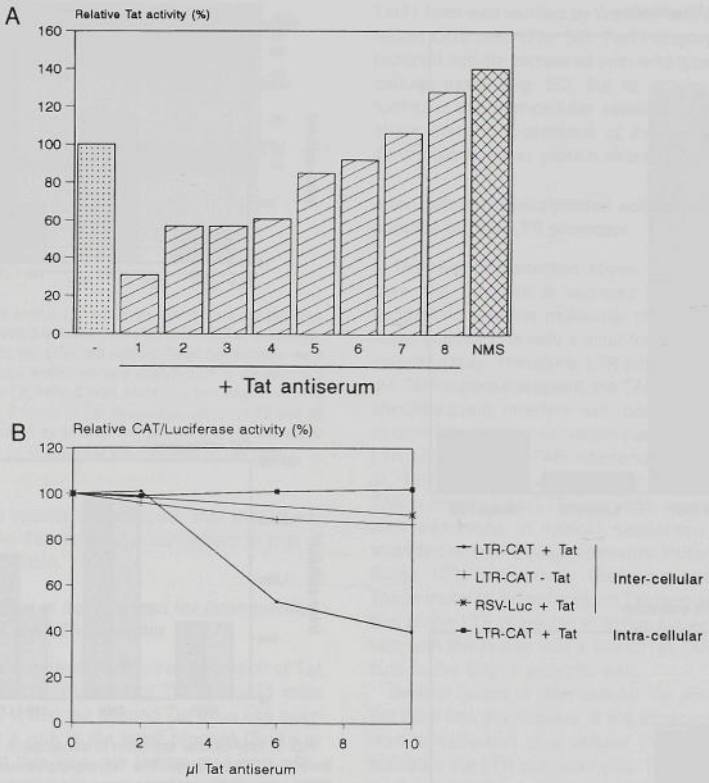



FIG. 8. Specific inhibition of *inter*-cellular LTR-activation by antiserum against Tat. (A) Eight Tat antisera were tested for inhibition in the COS-SupT1 coculture experiment. Tat-antisera 1–8 are specified under Materials and Methods. The Tat activity measured with a mock-treated sample (–) was set at 100%, corresponding to an 18-fold induction of LTR activity. Normal mouse serum (NMS) was included as an additional control. (B) The most potent antisera 1, 2, 3, and 4 were pooled and titrated in a coculture experiment with Tat-transfected COS cells and SupT1 cells that were cotransfected with LTR-CAT and RSV-Luc. A control coculture was performed with mock-transfected COS cells (LTR-CAT – Tat). In addition, the same antiserum mixture was titrated in the *intra*-cellular assay with LTR-CAT and Tat cotransfected SupT1 cells. The relative CAT/Luciferase activity measured in the absence of antiserum was set at 100% for each data set.

several attempts failed (Helland *et al.*, 1991; Marcuzzi *et al.*, 1992a). The ability of anti-Tat reagents to absorb extracellular Tat will depend on several variables, including antibody concentration and affinity. First, we tested eight Tat antisera (see Table 1) for inhibition of *inter*-cellular LTR-induction (Fig. 8A). Interestingly, several antisera reduced Tat-mediated LTR-transcription to 30–60% of the untreated cells (indicated by the – sign) or the control sample that was treated with normal mouse serum (NMS). Based on these results, we mixed the most potent sera (MAb 1, 2, 3, and 4) and performed a titration experiment. *Inter*-cellular Tat activity was gradually reduced by increasing the antiserum concentration (Fig. 8B, LTR-CAT + Tat). However, considerable Tat activity remained at the highest antiserum concentration.

To prove that the 60% reduction of Tat-induced LTR-transcription is meaningful and not caused by toxic effects of the MAb solution on the cells and their transcriptional capacity, several controls were included in this experiment. First, transcription was also measured on a cotransfected RSV-Luc plasmid with the Rous sarcoma virus promoter fused to the Luciferase reporter gene. In contrast to LTR-Tat-mediated CAT expression, RSV-promoted Luciferase synthesis was not affected by the Tat antiserum (Fig. 8B, RSV-Luc). This internal control ensures that no differences in transfection and cell harvest efficiencies are responsible for the observed effects. Second, we measured no effect of the Tat antiserum on the basal LTR promoter activity in SupT1 cells that were incubated with mock-transfected

TABLE 1

Tat Antibodies

| Tat antiserum (MAb/polyclonal) | Repository number | Isotype | Epitope | Reference, contributor |
|---|---|---|---|---|
| MAb 1 | NIH 1973 | Unknown | 45–64 | Drs. Krohn and Ovod |
| MAb 2 | | IgG1 | | Brake *et al.*, 1990b |
| MAb 3 | | IgG1 | | Brake *et al.*, 1990b |
| MAb 4 | | IgG1 | | Brake *et al.*, 1990b |
| MAb 5 | NIH 1976 | IgG1 | 77–86 | Drs. Krohn and Ovod |
| MAb 6 | NIH 1974 | IgG1 | 1–16 | Drs. Krohn and Ovod |
| Polyclonal 7 | NIH 705 | | | Hauber *et al.*, 1987 |
| MAb 8 | MRC 352 | IgG1a | 1–15 | Dingwall *et al.*, 1989 |

COS cells (Fig. 8B, LTR-CAT – Tat). Furthermore, we measured no inhibition by the Tat antisera in the standard *intra*-cellular Tat assay (Fig. 8B, LTR-CAT + Tat, *intra*-cellular), ruling out an aspecific detrimental effect of the antisera on SupT1 cells. This result also indicates that *intra*-cellular Tat action does not operate *via* an autocrine route (see discussion).

## DISCUSSION

Given the diversity of molecular models for *inter*-cellular Tat action (Fig. 1), we analyzed this mechanism in studies with mutant Tat proteins and reporter gene constructs with different promoter motifs. The results of this study are consistent with the idea that the Tat protein itself is (i) secreted, (ii) taken up by neighboring cells, (iii) localized to the nucleus, and (iv) involved in gene-activation (Fig. 1, route 2). Consistent with this direct pathway, we were able to partially block *inter*-cellular LTR-activation with Tat antibodies. A recent study reports that Tat antibodies can also block LTR-induction in the *intra*-cellular assay, suggesting an autocrine loop for normal Tat action (Zauli *et al.*, 1995). However, because the effect on non-LTR promoters was not tested in the latter study, a more general inhibitory effect on the cellular transcriptional capacity was not excluded. The results presented here indicate that Tat antisera do not block the standard *intra*-cellular mechanism, and even inhibition of *inter*-cellular Tat action was not absolute (this study and Helland *et al.*, 1991).

We tried to separate the ability of Tat to function in the *intra*- versus *inter*-cellular assay, but all Tat mutants tested behaved similarly in the two systems. Thus, sofar we failed to identify Tat "transport" domains that are required specifically for *inter*-cellular activity. In particular, our results indicate that the C-terminal Tat domain including the RGD motif is not critically involved in cellular uptake of the Tat protein, suggesting that integrin receptors do not play a role in the cellular uptake of Tat protein. The observation that the second Tat coding exon, including the RGD motif, is not required for activity in the *inter*-cellular assay is consistent with other published evidence. Tat-mediated delivery of heterologous proteins into cells (Fawell *et al.*, 1994) was efficient with peptides lacking the second coding exon sequences but retaining the basic domain of Tat (amino acids 37–72), suggesting that the RGD motif does not play a role in this process. Similarly, the effect of extracellular Tat on NF-κB binding activity was found with the exon-1 domain (amino acids 1–72) of Tat (Conant *et al.*, 1996) and binding of Tat to a 90-kDa cell surface protein was observed with the 49–57 domain (Weeks *et al.*, 1993). Furthermore, the RGD motif is only weakly conserved in different HIV-1 isolates and absent in the Tat proteins of the HIV-2 viruses and the different simian immunodeficiency viruses (SIV) (Jeang, 1995).

In summary, *inter*-cellular activation of the viral LTR promoter by Tat involves a transcriptional mechanism that is indistinguishable from that of *intra*-cellular *trans*-activation. Our experiments are consistent with the idea that Tat protein reaches the nucleus of the acceptor cell, where Tat will interact with TAR RNA on nascent HIV-1 transcripts and the cellular transcription machinery to trigger LTR transcription. We do not rule out that the *inter*-cellular effects of Tat on cellular genes operates through a distinct mechanism. For instance, whereas induction of HIV-1 gene expression was observed at high Tat concentrations (>100 ng/ml), cell-growth promoting effect of Tat was found to peak at 100- to 1000-fold lower Tat levels (Ensoli *et al.*, 1993). Whether the extracellular Tat route has any relevance *in vivo* remains to be determined, but there is some evidence to suggest that the paracrine route for Tat-mediated LTR-activation is more important than previously considered. First, *inter*-cellular Tat action in cocultured lymphocytes requires cell–cell contact via the CD4-gp120 interaction (Marcuzzi *et al.*, 1992a) and in this system one Tat-producing cell activates the LTR-promoter in 100–1000 target cells (Marcuzzi *et al.*, 1992b). Second, antibodies to Tat were found to significantly inhibit HIV-1 replication in tissue culture, suggesting that extracellular Tat is important for viral replication (Steinaa *et al.*, 1994; Re *et al.*, 1995). Third, antibodies that recognize Tat protein are detectable in the sera of the majority of seropositive patients (Krone *et al.*, 1988), suggesting that Tat is released in the course

of HIV infection, either by cell death or active secretion. Furthermore, low antibody titers to the HIV-1 regulatory proteins (Tat, Rev, Nef) in patients may be associated with a relatively rapid progression to AIDS (Reiss *et al.*, 1991; Re *et al.*, 1995).

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# Chapter 4

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# On the Role of the Second Coding Exon of the HIV-1 Tat Protein in Virus Replication and MHC Class I Downregulation

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## ABSTRACT

**Tat is an essential protein of human immunodeficiency virus type 1 (HIV-1) and activates transcription from the viral long terminal repeat (LTR) promoter. The *tat* gene is composed of two coding exons of which the first, corresponding to the N-terminal 72 amino acid residues, has been reported to be sufficient for its transcription function. We introduced a stop codon at the end of the first Tat-coding exon in an expression vector that produces a truncated 71-amino acid Tat protein. This Q72stop mutant displays reduced transcriptional activity of approximately 54% in transient LTR-CAT transfection assays. To test the contribution of the second Tat-coding exon to virus replication, the Q72stop mutation was also introduced in the infectious pLAI molecular clone. The effect on virus replication was analyzed in primary cells and in a transformed T cell line. The fitness of the mutant virus was calculated to be approximately 75% compared with the wild-type control. Thus, a small contribution of the C-terminal Tat domain to viral fitness was measured. It has been proposed that the second Tat-coding exon is involved in transcriptional downregulation of the MHC class I gene of the infected host cell. Cell surface expression of the MHC protein was analyzed in T cells infected with the wild-type LAI virus and the replication-competent Q72stop mutant. MHC expression was transiently reduced on infection with either virus, indicating that the second Tat-coding exon is not involved in this downregulation.**


## INTRODUCTION

THE Tat PROTEIN of the human immunodeficiency virus type 1 (HIV-1) is a potent *trans*-activator of transcription from the viral promoter and is essential for viral replication (reviewed in Refs. 1 and 2). The full-length Tat protein is synthesized from a fully spliced mRNA species and is 86–101 amino acids in length, depending on the viral isolate. Late in the replication cycle, the viral Rev protein shifts the balance toward unspliced mRNAs that express a "one-exon" 72-amino acid Tat protein. Both forms of Tat can efficiently activate the viral long terminal repeat (LTR) promoter in transient transfection assays,[3–7] although a small difference in activity has been reported for a chromosomally integrated LTR promoter.[8] We tested the contribution of the second Tat-coding exon to *trans*-activation and virus replication by introduction of a premature stop codon, yielding a Tat protein of 71 amino acids. This truncated Tat mutant, Q72stop, demonstrated a partial defect in *trans*-activation of an LTR-CAT reporter in transiently transfected T cells. Virus replication was measured in sensitive cocultures with both viruses, demonstrating a small, but significant contribution of the C-terminal Tat domain to viral fitness. This result is consistent with the conservation of the second Tat-coding exon in natural HIV and SIV isolates.

A variety of cellular genes have been reported to be modulated by Tat in HIV-infected cells (reviewed in Ref. 9). These effects can reach bystander cells because Tat can act in an intercellular manner, e.g., the protein is secreted by producer cells and taken up by other cells.[10–13] The ability of HIV-1 Tat to repress MHC class I gene transcription potentially contributes to viral persistence.[14] Remarkably, this MHC repression has been reported to be specific for the "two-exon" 86-amino acid form of Tat.[14] In this study, we analyzed Tat-mediated repression of host MHC class I genes with the replication-competent HIV-1 mutant that does not express the second Tat-coding exon. Although MHC cell surface expression was transiently reduced in infected cells, we measured no significant difference between wild-type LAI virus and the Q72stop mutant.

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## MATERIALS AND METHODS

### *Cells, transfection, and infection*

COS, HeLa, and HLCD4-CAT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). HLCD4-CAT is a HeLa-derived cell line with an integrated LTR-CAT construct.[15] The SupT1 T cell line was grown in RPMI 1640 medium containing the same supplements. The isolation of peripheral blood mononuclear cells (PBMCs), phytohemagglutinin (PHA) stimulation, and culturing were performed as described.[16] Transient transfection of adherent cells was performed on a subconfluent layer in 60-mm dishes by the DEAE-dextran protocol with cesium-purified DNA.[17] The amount of LTR-CAT plasmid was optimized for each cell line and pTat was added in the linear range of transcriptional activation. We used either 0.1 μg of pcDNA3-Tat and 1 μg of LTR-CAT (COS cells), 1 μg of of pcDNA3-Tat and 1 μg of LTR-CAT (HeLa), or 2 μg of pcDNA3-Tat (HLCD4-CAT). SupT1 T cells ($5 \times 10^6$) were transfected transiently by electroporation with 2.5 μg of pcDNA3-Tat and 1 μg of LTR-CAT reporter construct. SupT1 T cells and PBMCs were electroporated with 1 and 2 μg, respectively, of the HIV-1 molecular clone pLAI. After PBMC transfection, $0.5 \times 10^6$ fresh cells were added to support viral replication. Supernatants were collected and infectious virus was titered on MT-2 cells as described previously.[18] In the coculture experiment, an equimolar mixture of wild-type pLAI and pLAI Q72stop (0.5 μg each) was transfected. At the peak of viral production (large Env-induced syncytia), 0.1 μl of supernatant was used to infect fresh SupT1 cells. At the same time, infected cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and stored at −70°C for sequence analysis of integrated HIV-1 genomes.

### *Plasmids and site-directed mutagenesis*

The expression vector pcDNA3-Tat was described previously.[19] This plasmid contains the HIV-1 LAI *tat* gene under the transcriptional control of the cytomegalovirus (CMV) immediate-early promoter and was generated by subcloning a 3.2-kb *Eco*RI/*Xho*I fragment of CMV-Tat[20] into the corresponding sites of pcDNA3 (Invitrogen, San Diego, CA). The 0.6-kb *Asp*718 fragment, containing the first Tat-coding exon, was subcloned in the *Asp*718 site of M13mp18. Single-stranded phage DNA with the Tat-coding (+)strand was generated. This template was used in a mutagenesis reaction with the Bio-Rad (Hercules, CA) Muta-gene kit[21] and the (–)strand mutagenic primer (Q72stop, 5′ ATGTACTACTTACTACTTTGATAGAGA 3′; mismatching nucleotide underlined). The mutation was verified by dideoxy sequencing and the mutant gene was recloned as an *Asp*718 fragment in pcDNA3-Tat. The LTR-CAT plasmid was

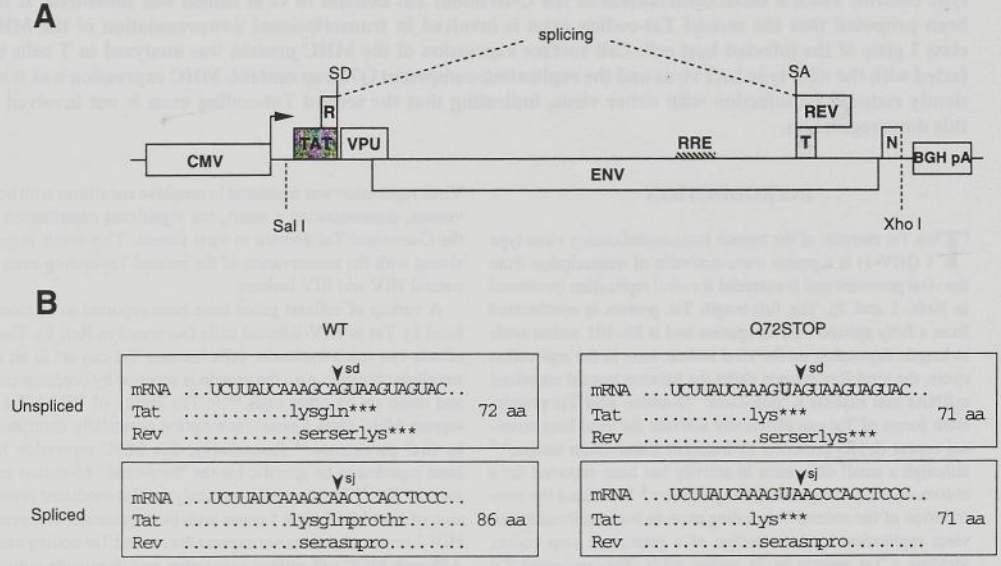


**FIG. 1.** (**A**) Schematic of the Tat expression vector pcDNA3-Tat. Shown are the vector-derived cytomegalovirus (CMV) immediate-early promoter (transcription start site is marked by an arrow) and the polyadenylation signal of the bovine growth hormone gene (BGH pA). The *Sal*I and *Xho*I restriction sites denote the borders of the subgenomic HIV-1 LAI insert. Tat and T, first and second coding exons of Tat; R and Rev, first and second coding exons of Rev; N, truncated Nef open reading frame. The splicing signals (SD and SA) and the Rev response element (RRE) are indicated. (**B**) Shown are the Tat and Rev coding potentials of the wild type (*left*) and of the Q72stop mutant (*right*), both in unspliced (*top*) and spliced (*bottom*) form. The C-to-U base substitution in Q72stop is outlined; this mutation creates a stop codon in both unspliced and spliced mRNA. Indicated is the splice donor (sd) in unspliced transcripts and the splice junction (sj) in spliced transcripts, stop codons are marked by triple asterisks (***). Q72stop produces a 71-residue Tat protein from both unspliced and spliced RNA.

described previously (pBlue-3'LTR-cat).[22] The HIV-1 molecular clone pLAI was generously provided by Keith Peden (Food and Drug Administration, Bethesda, MD). The Q72stop mutation was introduced in pLAI by exchange of the 2.8-kb *Sall*/*Bam*HI fragment of pcDNA3-Tat with the corresponding fragment of pLAI.

### *CAT assay and CA-p24 ELISA*

SupT1 cells were collected by centrifugation 3 days post-transfection and adherent cells were washed in PBS and harvested by trypsinization.[13] The cells were washed with PBS and resuspended in 200 $\mu$l of 0.25 M Tris-HCl (pH 8.0). Cell lysates were prepared by three cycles of freeze–thawing (−80°C/37°C) and chloramphenicol acetyltransferase (CAT) enzyme assays were performed by the phase-extraction protocol.[23] Capsid protein CA-p24 levels were determined by enzyme-linked immunosorbent assay (ELISA) of supernatant samples from virus-infected cell cultures.[16]

### *Proviral DNA sequence analysis*

Proviral *tat* sequences were amplified in a polymerase chain reaction (PCR)[24] with total cellular DNA from infected cells and a primer pair encompassing the first Tat-coding exon (L5, sense, 5′ CATGCGGCCGCTTCTACAAAACTGCTGTTT 3′; and WS3, antisense, 5′ TACTTAAGAACTAGGGTATTTGACTAAT 3′. The PCR product was sequenced with a primer complementary to HIV-1 sequences upstream of the *tat* gene (TAT-AUG, 5′ ATGGAGCCAGTAGATCCTAG 3′). The Dye Dideoxy Terminator cycle sequencing kit (Amersham, Arlington Heights, IL) was used in combination with an Applied Biosystems (Foster City, CA) automated DNA sequencer. This population-based sequence was used to calculate the relative fitness according to Ref. 25. In brief, the relative fitness of the Tat-mutated virus was approximated from the equation $p/q = [p(0)/q(0)] \times (\text{fitness})^T$, where $p$ is the proportion of mutant virus, $q$ is the proportion of the wild-type virus, 0 indicates time zero, and $T$ is the time in viral generations (2 days/generation).

### *Determination of MHC class I expression on HIV-infected primary CD4$^+$ T lymphocytes*

Peripheral blood mononuclear cells (PBMCs) of HIV-seronegative blood donors were separated on a Ficoll-Hypaque (Pharmacia Biotech, Freiburg, Germany) density gradient. Monocytes were depleted by plastic adherence and CD4$^+$ T cells were positively selected by Dynabeads M450 CD4 (Dynal, Hamburg, Germany). Enriched CD4$^+$ T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics, and stimulated with phytohemagglutinin (PHA-P, 2.5 $\mu$g/ml; Sigma Chemicals, St. Louis, MO) for 2 days prior to infection. Stimulated cells ($3 \times 10^6$) were infected with HIV-1 LAI and HIV-1 LAI Q72stop at a multiplicity of infection (MOI) of 1. To analyze MHC class I expression, infected cells were stained daily with W6/32 (anti-HLA-I; Dako, Hamburg, Germany) as first antibody, and with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (Sigma) as second antibody. Uninfected cells were stained accordingly and used as reference. The fluorescence intensity of 20,000 cells was accumulated by using a FACSort (Becton Dickinson, Heidelberg, Germany) and data were analyzed with the software Cell Quest (Becton Dickinson). The percentage of MHC class I downregulation was calculated as mean fluorescence of infected cells/mean fluorescence of uninfected cells × 100. This value varied less then 5% between independent experiments.

## RESULTS

### *The role of the second Tat-coding exon in LTR transcription and viral replication*

To test the contribution of the second Tat-coding exon to viral transcription, we designed a Tat mutant that expresses exclusively the first exon form of the Tat protein. The pcDNA3-Tat expression vector (Fig. 1A) was mutated to synthesize a truncated form of Tat. A stop codon was generated by a C-to-U point mutation at codon 72, which is at the end of the first Tat-coding exon (mutant Q72stop; Fig. 1B). The mutation was designed such that a stop codon is present on both the unspliced and spliced mRNA species (UAG and UAA, respectively). Furthermore, the point mutation does not affect the GU dinucleotide that is critical for splice donor (SD) activity, nor does the mutation affect the underlying Rev coding information because both the AGC (wild-type) and AGU (mutant) codons encode serine. Expression of the truncated Q72stop protein was verified on Western blots (results not shown; see also Ref. 13), indicating that the Tat mutant is expressed as a stable protein.

The Q72stop mutant demonstrated reduced *trans*-activation activity compared with wild-type Tat in transient LTR-CAT transfection assays performed in the SupT1 T cell line (54%, Table 1). Similar results were obtained in HeLa cells, and in HLCD4-CAT cells that contain an integrated LTR-CT reporter (Table 1). Remarkably, we consistently measured increased transcriptional activity (142%) for the short Tat form in COS cells. To assess directly the role of the second Tat-coding exon in viral replication, the stop codon mutation was introduced into the molecular clone pLAI. Viral replication was monitored after transfection of the SupT1 T cell line and PBMCs by measuring CA-p24 antigen production in the culture medium (Fig. 2). No significant difference in viral replication potential was measured between the wild-type virus and Q72stop mutant in both the transformed and primary cell type.

The results presented here clearly indicate that the Q72stop mutant is replication competent. However, replication assays cannot easily measure small differences in replication rates. Therefore, we performed a direct competition experiment by

TABLE 1. TRANSCRIPTIONAL ACTIVITY OF THE Q72stop Tat MUTANT

| *Cell type* | *Activity*[a] (%) |
|---|---|
| SupT1 | 54 |
| HeLa | 43 |
| HLCD4-CAT | 51 |
| COS | 142 |

[a]Expressed as the percentage transcriptional activity, compared with that of wild-type Tat protein (set at 100%). The values represent the average of three independent transfections per cell line that were performed on different occasions.

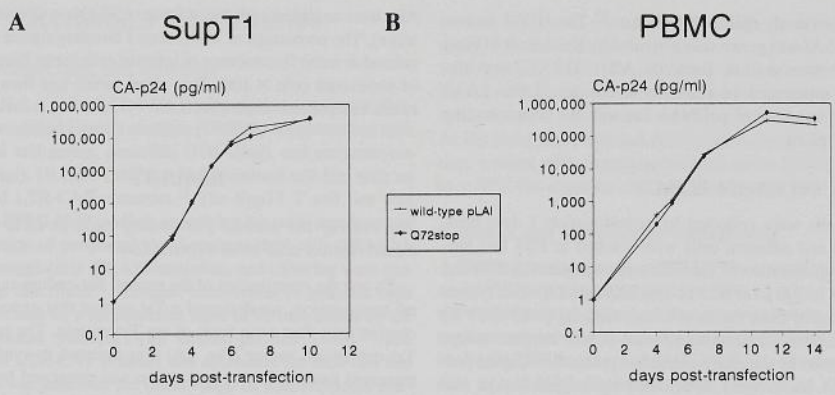


**FIG. 2.** Viral replication of wild-type HIV-1 and of the Q72stop mutant in the SupT1 T cell line (**A**) and peripheral blood mononuclear cells (**B**). Virus production in the culture supernatant was measured by CA-p24 ELISA several days posttransfection. Similar results were obtained in independent infections performed on different occasions.

coculture of the two viruses, in which the faster replicating virus will outgrow the less fit variant.[26] Such an experiment is internally controlled because the two viruses replicate in the same culture flask. Furthermore, this experiment is deterministic and not stochastic, because relatively large virus populations are used, and the same result is obtained in independent cocultures.[26] An equimolar mixture of the wild-type and mutant pLAI plasmids was transfected into SupT1 cells and virus replication was continued for several weeks by passage of the culture supernatant onto uninfected SupT1 cells. Samples of infected cells were taken for DNA isolation, PCR amplification of proviral Tat sequences, and population-based sequence analysis. The sequence of the genome surrounding codon 72 of the input DNA sample (day 0) and of the cellular DNA isolated on days 6, 13, and 22 posttransfection is shown in Fig. 3A, with quantitation of the codon 72 composition provided in Fig. 3B. We observed gradual outgrowth of the wild-type virus, indicating that the second Tat-coding exon contributes to optimal viral replication. The fitness loss of the Q72stop virus was approximated to be 25%, which is consistent with the re-

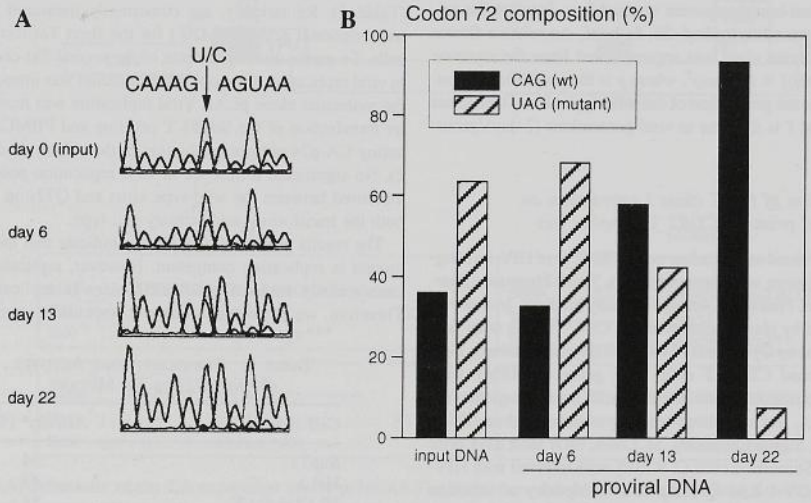


**FIG. 3.** Competition between wild-type HIV-1 LAI virus and the Q72stop mutant. An equimolar mixture of the two HIV plasmids was transfected into SupT1 cells and the culture was maintained for several weeks. Proviral *tat* sequences were PCR amplified from infected cells at several times posttransfection and sequenced. (**A**) Sequence analysis in the region encompassing codon 72 of the *tat* gene. As a control, we sequenced the DNA mixture used for transfection (day 0). The double U/C signal (see arrow) demonstrates the presence of a wild-type/Q72stop mixture. (**B**) Quantitation of codon 72 composition, which was calculated from the peak volumes of the U/C signals at the first nucleotide position of *tat* codon 72.

duced transcriptional activity measured for this Tat mutant in the SupT1 T cell line.

### *The role of the second Tat-coding exon in MHC downregulation*

Previously it was shown that two-exon Tat represses the MHC class I gene promoter in transiently transfected human HeLa cells.[14] However, the significance of this repression for MHC class I expression in HIV-infected primary cells is unclear. Therefore surface expression of MHC class I was analyzed on primary $CD4^+$ T lymphocytes after infection with wild-type HIV-1 LAI or the Q72stop mutant. Both HIV-1 variants transiently downregulated MHC class I expression (Fig. 4). Thus, the C-terminal Tat domain is not critical for the MHC downregulation in infected cells. Maximal percentage of downregulation was between 25 and 30% on day 3 to 4 after infection. The magnitude of this suppression was comparable to previous observations with HIV-1 LAI and HIV-1 NL4-3 in PBMCs and $CD4^+$ T cell lines.[27,28] This MHC suppression is most likely due to the action of the viral Nef protein.[29,30]

## DISCUSSION

The contribution of the C-terminal Tat domain to virus replication and MHC class I downregulation was analyzed. To do so, a stop codon was introduced at codon position 72 of the *tat* gene, both in a Tat-expression vector and in the context of the infectious LAI molecular plasmid. Reduced Tat activity was measured in transient LTR-CAT assays (54% activity in SupT1 cells), but we did not measure a gross difference in viral replication rate in this T cell line. Several of the HIV-1 "accessory functions" are less critical for replication in transformed T cell lines than in primary cells.[31] We therefore also performed replication studies with the Tat mutant in primary cells, but again little difference in replication rate was measured. A sensitive coculture experiment was subsequently used to demonstrate that the Q72stop mutant is less fit than the wild-type virus. The rapidity of outgrowth of the wild-type virus indicated a significant replication defect of the Tat mutant of approximately 25%. The combined results of this analysis and a previous study[32] suggest that the second Tat-coding exon contributes to the replication capacity of HIV-1.

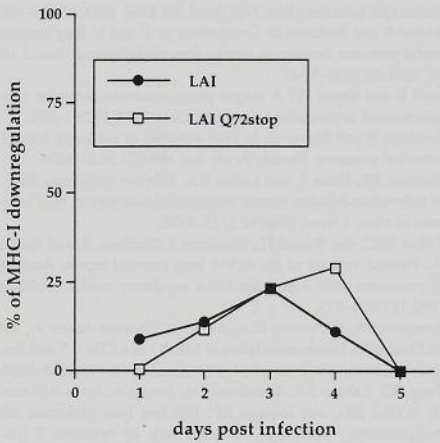


**FIG. 4.** HIV-1-mediated downregulation of MHC class I surface expression on primary $CD4^+$ T lymphocytes. PHA-stimulated primary $CD4^+$ T lymphocytes were infected with HIV-1 LAI (filled circles) and HIV-1 LAI Q72stop (open squares) at an MOI of 1. MHC class I surface expression was determined by flow cytometry 1 to 5 days postinfection and is given as a percentage of MHC-I downregulation compared with HIV-uninfected cells. This experiment was repeated twice in independent infections and the results were identical.

Although reduced Tat activity was measured for the Q72stop mutant in most cell types, including T cells, slightly improved activity was consistently measured in the simian virus 40 (SV40)-transformed monkey kidney COS cell line. Interestingly, we have described a similar cell type-dependent phenomenon for HIV-1 LTR promoter variants with mutations in the TAR hairpin that forms the binding site for the Tat protein.[33] The exceptional behavior of COS cells may be caused by high-level replication of our Tat expression plasmid that contains the SV40 origin of replication. Alternatively, there may be differences in the cellular cofactors among different cell types. Whatever the reason for the exceptional results obtained in COS cells, we do not recommend the use of these cells in HIV-1 transcription studies. The cell type-dependent variation in Tat activity of the Q72stop mutant may also explain some of the contradictory results obtained with similarly truncated Tat mutants in previous studies.[3–6,32,34–38]

The observation that HIV-1 replicates efficiently with a truncated Tat protein allowed us to test the effect of the second Tat-coding exon on downregulation of gene expression of the host MHC class I gene.[14] Different viruses use distinct strategies to evade the immune response of the infected host (reviewed in Ref. 39). For instance, viruses may escape both antibodies and T cell recognition by mutation of the major epitopes (antigenic drift), or may selectively inhibit the expression of host cell molecules, such as the MHC class I complex, that are critically involved in the immune response. MHC class I consists of a heavy ($\alpha$) chain and light ($\beta_2$-microglobulin) chain that assemble in the endoplasmic reticulum (ER). On binding of degraded viral peptides that are shunted from the cytosol to the ER by the TAP1 and TAP2 transporter proteins, the MHC complex is transported across the Golgi to the cell membrane. Viral infections can impede the constitutive pathway of MHC class I expression at several levels.[39] For instance, cells infected with adenovirus and human cytomegalovirus demonstrate retention and enhanced degradation of the MHC class I complex within the ER, respectively. It has been reported that the HIV-encoded Tat *trans*-activator protein specifically represses the promoter activity of the MHC class I gene.[14,40] However, downregulation of MHC class I cell surface expression in primary $CD4^+$ T lymphocytes infected with the wild-type or Q72stop mutant HIV-1 is transient and of comparable magnitude. Thus, the C-terminal domain of Tat is not a dominant suppressor of MHC class I expression. The reduced level of MHC expression was comparable to that observed for other HIV-1 variants following *in vitro* infections[27,41,42] and in $CD4^+$ T lymphocytes of

HIV-1-infected patients.[43] This level of downregulation was shown not to be sufficient for effecting HIV-specific cytotoxic T lymphocyte (CTL)-mediated target cell lysis.[28]

In conclusion, we measured a replication advantage of HIV-1 with a full-length Tat protein. This contribution to full transcriptional activity and viral fitness explains the absolute conservation of the second Tat-coding exon in all HIV–SIV viruses. The second Tat-coding exon is also used in the other two reading frames to encode part of the Rev and Env proteins, and these two functions may be more critical for the virus than encoding a particular C-terminus of the Tat protein. It is obvious that the presence of three overlapping reading frames puts severe constraints on the evolutionary flexibility of this part of the HIV-1 genome.

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# Chapter 5

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# Determination of the Minimal Amount of Tat Activity Required for Human Immunodeficiency Virus Type 1 Replication

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The Tat protein of human immunodeficiency virus type 1 (HIV-1) is a potent *trans*-activator of transcription from the viral LTR promoter. Previous mutagenesis studies have identified domains within Tat responsible for binding to its TAR RNA target and for transcriptional activation. The minimal Tat activation domain is composed of the N-terminal 48 residues, and mutational analyses identified a cluster of critical cysteines. The importance of four highly conserved aromatic amino acids within the activation domain has not been thoroughly investigated. We have systematically substituted these aromatic residues (Y26, F32, F38, Y47) of the HIV-1 LAI Tat protein with other aromatic residues (conservative mutation) or alanine (nonconservative mutation). The activity of the mutant Tat constructs was measured in different cell lines by transfection with a LTR–CAT reporter plasmid. The range of transcriptional activities measured for this set of Tat mutants allowed careful assessment of the level of Tat activity required for optimal viral replication. To test this, the mutant Tat genes were introduced into the pLAI infectious molecular clone and tested for their effect on virus replication in a T-cell line. We found that a twofold reduction in Tat activity already affects viral replication, and no virus replication was measured for Tat mutants with less than 15% activity. This strict correlation between Tat activity and viral replication demonstrates the importance of the Tat function to viral fitness. Interestingly, a less pronounced replication defect was observed in primary cell types. This finding may correlate with the frequent detection of proviruses with Tat-inactivating mutations in clinical samples. 

## INTRODUCTION

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is essential for viral replication. Full-length Tat is encoded by two exons on a spliced transcript and is 86–101 amino acids in length, depending on the viral isolate. Tat is directed to the nucleus by a basic nuclear localization domain and *trans*-activates transcription from the viral promoter located in the 5′ long-terminal-repeat (LTR) region of the HIV-1 genome. The binding site for Tat in the promoter region is formed by an RNA stem–loop structure present at the 5′ end of all HIV mRNAs, the *trans*-acting responsive (TAR) element (Berkhout *et al.*, 1989; Dingwall *et al.*, 1989). Whereas Tat binds a three-nucleotide bulge in the TAR hairpin (Berkhout and Jeang, 1989; Dingwall *et al.*, 1990), the essential TAR loop has been proposed to contribute to the *trans*-activation mechanism by binding of a cellular cofactor (Sheline *et al.*, 1991; Wu *et al.*, 1991). In addition, cofactors may interact with the Tat protein itself (Fridell *et al.*, 1995; Kashanchi *et al.*, 1994; Desai *et al.*, 1991; Veschambre *et al.*, 1995; Zhou and Sharp, 1995; Yu *et al.*, 1995a,b; Wu-Baer *et al.*, 1995; Gutheil *et al.*, 1994; Jeang *et al.*, 1993; Herrmann and Rice, 1993; Zhou and Sharp, 1996; Parada and Roeder, 1996). Several TAR- and Tat-binding proteins have been identified, but it is currently unknown which cellular proteins participate in Tat-mediated transcriptional activation.

On the basis of Tat mutational analyses and phylogenetic comparisons of protein sequences from different HIV-1 isolates, a domain model has been proposed for the Tat protein (Fig. 1A) (Kuppuswamy *et al.*, 1989). Tat was arbitrarily divided into an acidic N-terminal domain, a cysteine-rich domain of 16 amino acids including 7 cysteine residues, a highly conserved core domain, and a basic domain consisting of a stretch of positively charged amino acids. The C terminus of the protein spans the splice junction and is rich in glutamine residues. The first coding exon of Tat (amino acids 1–72) is sufficient for *trans*-activation of the HIV-1 promoter (Seigel *et al.*, 1986; Hauber *et al.*, 1989; Ruben *et al.*, 1989; Kuppuswamy *et al.*, 1989; Garcia *et al.*, 1988). Positively charged amino acids of the basic domain (residues 49–57) are essential both for nuclear localization of the Tat protein and for binding to the three-nucleotide bulge in the TAR RNA hairpin. Site-directed mutagenesis studies revealed that the minimal Tat activation domain is located between residues 1 and 48 (Kuppuswamy *et al.*, 1989; Garcia *et al.*, 1988; Rice and Carlotti, 1990; Ruben *et al.*, 1989; Sadaie *et al.*, 1988). Point mutations that cause a severe Tat defect, but retain TAR RNA binding activity, cluster in the cysteine-rich and core domains (Jeang, 1995). These domains could be involved in the binding of cofactors. One notable feature of the Tat activation domain is the cysteine-rich cluster, which has hampered

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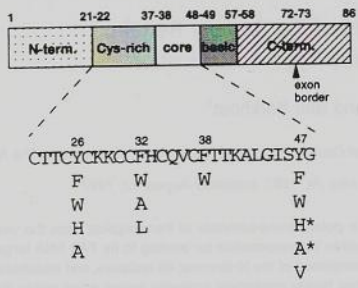
A




B

| | Rev start | Rev translation | Tat form (amino acids) |
|---|---|---|---|
| Y47 (wt) | CUCCU AUG GCA | + | 72>86 |
| Y47F | ....U UCG ... | - | 86>72 |
| Y47W | ....U GGG ... | - | 86>72 |
| Y47H1 | ....C AUG ... | + | 72>86 |
| H2 | ....C ACG ... | - | 86>72 |
| Y47A1 | ....G CCG ... | - | 86>72 |
| A2 | ....G CUG ... | - | 86>72 |
| Y47V | ....G UCG ... | - | 86>72 |

FIG. 1. Domain structure of the HIV-1 Tat protein and mutations introduced in the activation domain. (A) The arbitrary domain structure is that according to Kuppuswamy *et al.* (1989). Amino acid (aa) numbers at the domain borders are depicted, and a dotted line in the C-terminal domain represents the exon border. The aa sequence of the Cys-rich and core domains of HIV-1 LAI Tat is presented in single-letter code. Aromatic residues 26, 32, 38, and 47 were mutated as indicated (Y = tyrosine, F = phenylalanine, W = tryptophan, H = histidine, A = alanine, L = leucine, V = valine). Two forms of mutants, Y47H and Y47A (marked with asterisks), were generated with synonymous codons. (B) Tat codon 47 overlaps the Rev translation initiation codon. The Rev initiation codon AUG is underlined in the wild-type sequence. All Tat Y47 mutants except Y47H1 alter the Rev start codon, thereby inhibiting Rev translation. This leads to efficient splicing and synthesis of the full-length 86-aa Tat protein.

structural studies because of oxidation and protein insolubility. Six of the seven cysteine residues were shown to be critically important for *trans*-activation activity. These residues may be involved in binding of a divalent cation (Frankel *et al.*, 1988) or intrachain disulfide bond formation (Koken *et al.*, 1994a). The core domain is the phylogenetically most conserved part of the protein and is thought to contribute to binding of Tat to the TAR element (Churcher *et al.*, 1993).

To date biophysical studies have provided little detail on the Tat protein structure. Two structured regions were identified by NMR: a hydrophobic core (residues 32–47) and the C-terminal glutamine-rich domain (residues 60–76), surrounded by the highly flexible cysteine-rich and basic domains (Bayer *et al.*, 1995). NMR/CD studies on Tat peptides suggest an $\alpha$-helical conformation for the stretch of basic residues constituting the nuclear localization/RNA-binding domain (Mujeeb *et al.*, 1994) and an amphipatic $\alpha$-helix for the core region (Loret *et al.*, 1991). In a separate study with the Tat 1–37 domain fused to the $\alpha$-amylase inhibitor Tendamistat, this Tat domain was shown to consist of an extended domain (residues 1–21) and a coiled domain (residues 22–37) (Freund *et al.*, 1995). To gain further insight into the structure/function of the HIV-1 Tat protein, we decided to analyze this protein in further detail by site-directed mutagenesis. In this study, we focus on four conserved aromatic amino acid residues within the activation domain (Fig. 1A). These residues were substituted either by other aromatic amino acids (conservative change) or by nonaromatic residues (nonconservative change). The activity of the mutant Tat proteins was measured by transient cotransfection of a Tat expression vector and a LTR–CAT reporter construct in different cell lines. This set of Tat mutants exhibited a wide range of activities, and was subsequently introduced into an infectious molecular clone to measure the minimal Tat activity required for virus replication.

## MATERIALS AND METHODS

### Cells, transfection, and infection

COS, HeLa, and HLCD4-CAT cells were maintained in DMEM medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). HLCD4-CAT is a HeLa-derived cell line with an integrated LTR–CAT construct (Ciminale *et al.*, 1990). The SupT1 T-cell line was grown in RPMI 1640 medium containing the same supplements. Isolation of peripheral blood mononuclear cells (PBMCs), phytohemagglutinin (PHA) stimulation, and culturing were performed as described (Back *et al.*, 1996).

Transient transfection of adherent cells was performed in subconfluent 60-mm dishes by the DEAE-dextran protocol with cesium-purified DNA (Berkhout and Jeang, 1989). The amount of LTR-CAT plasmid was optimized for each cell line and pTat was added in the linear range of transcriptional activation. We used either 0.1 $\mu$g pcDNA3–Tat and 1 $\mu$g LTR–CAT (COS cells), 1 $\mu$g of pcDNA3–Tat and 1 $\mu$g LTR–CAT (HeLa), or 2 $\mu$g of pcDNA3–Tat (HLCD4-CAT). Five million SupT1 T cells were transfected transiently by electroporation with 1 $\mu$g of pcDNA3–Tat and 2.5 $\mu$g of LTR–CAT reporter construct (Koken *et al.*, 1994a). SupT1 T cells and PBMC were electroporated with 1 and 2 $\mu$g, respectively, of the HIV-1 molecular clone pLAI, and 0.5 $\times$ $10^6$ fresh cells were added after transfection to support viral replication.

### Plasmids and site-directed mutagenesis

The expression vector pcDNA3–Tat, with the HIV-1 LAI Tat gene under control of the cytomegalovirus (CMV) immediate-early promoter, was generated by subcloning a 3.1-kb *Eco*R1/*Xho*1 fragment from CMV-Tat (Koken *et*

*al.*, 1994b) into the corresponding sites in pcDNA3 (Invitrogen). For mutagenesis, the 0.6-kb *Asp*718 fragment with the first Tat coding exon was subcloned in the *Asp*718 site of M13mp18. Single-stranded phage DNA of M13mp18Tat containing the coding (+) strand of the Tat gene was used as template in the mutagenesis reaction with the Bio-Rad Muta-gene kit (Kunkel, 1985). The mutations were verified by dideoxy sequencing and the mutant genes were recloned as *Asp*718 fragment in pcDNA3–Tat. The following (–) strand mutagenic primers were used (mismatching nucleotides underlined):

Y26F 5′ ACACTTTTTACAGAAGCAAGTGGTACA 3′,
Y26W 5′ ACACTTTTTACACCAGCAAGTGGTACA 3′,
Y26H 5′ ACACTTTTTACAGTGGCAAGTGGTACA 3′,
Y26A 5′ ACACTTTTTACAGGCGCAAGTGGTACA 3′,
F32W 5′ AACTTGGCAATGCCAGCAACACTTTTT 3′,
F32A 5′ AACTTGGCAATGGGCGCAACACTTTTT 3′,
F32L 5′ AACTTGGCAATGGAGGCAACACTTTTT 3′,
F38Y 5′ GGCTTTTGTTGTATAACAAACTTGGCA 3′,
F38W 5′ GGCTTTTGTTGTCCAACAAACTTGGCA 3′,
F38A 5′ GGCTTTTGTTGTAGCACAAACTTGGCA 3′,
Y47F 5′ CTTCTTCCTGCCGAAGGAGATGCCTAA 3′,
Y47W 5′ CTTCTTCCTGCCCCAGGAGATGCCTAA 3′,
Y47H1 5′ CTTCTTCCTGCCATGGGAGATGCCTAA 3′,
Y47H2 5′ CTTCTTCCTGCCGTGGGAGATGCCTAA 3′,
Y47A 5′ CTTCTTCCTGCCGGCGGAGATGCCTAA 3′.

The mutants Y47A2 and Y47V used in this study were not designed, but were fortuitously generated in the mutagenesis reaction with primer Y47A. Mutants F38Y and F38A were constructed in M13mp18Tat, but repeatedly resisted subcloning into pcDNA3–Tat. The reason for this problem is currently unknown. The LTR–CAT plasmid used was described previously (pBlue-3′ LTR-cat) (Klaver and Berkhout, 1994). The HIV-1 molecular clone pLAI was generously provided by Dr. Keith Peden (Peden *et al.*, 1991). These Tat mutations were introduced in this infectious construct by exchange of the 2.8-kb *Sal*1/*Bam*H1 fragment of the pcDNA3–Tat plasmids with the corresponding fragment of pLAI.

### CAT assay and CA-p24 ELISA

For CAT assays, the adherent cell types were washed in PBS and harvested by trypsinization 3 days post-transfection. (Verhoef *et al.*, 1996). SupT1 cells were collected by centrifugation, washed with PBS, and resuspended in 200 $\mu$l of 0.25 *M* Tris–HCl (pH 8.0). Cell lysates were prepared by three cycles of freeze/thawing (–80°/37°) and CAT assays were performed by the phase-extraction protocol (Seed and Sheen, 1988). CA-p24 levels were determined by ELISA on supernatant samples from virus-infected cell cultures (Back *et al.*, 1996).

### Western blotting

Detection of Tat protein expression was carried out as follows. Subconfluent COS cells in 70-cm$^2$ dishes were transfected with 10 $\mu$g of the individual pcDNA3–Tat constructs by the DEAE-dextran transfection method (Berkhout and Jeang, 1989). Cells were washed 2 days post-transfection with PBS and lysed in 1 ml (2× concentrated) SDS sample buffer. The lysate was homogenized by shearing through a syringe needle (21 gauge) to reduce viscosity. The samples were separated by electrophoresis on a 20% SDS–PAA gel, which was subsequently blotted on a nitrocellulose filter (Millipore). PAGE and Western blotting were performed essentially as described (Sambrook *et al.*, 1989). Tat protein was detected with Tat-specific mouse monoclonal antibodies and goat anti-mouse alkaline phosphatase conjugate. The blots were stained with BCIP/NBT (Sigma). The monoclonal antibodies (Nos. 2 and 4) used in the different Western blot experiments were generously provided by Dr. Christine Debouck (Brake *et al.*, 1990).

## RESULTS

### Mutation of conserved aromatic residues in the HIV-1 Tat activation domain

We constructed an expression vector that allows high-level expression of the full-length HIV-1 Tat protein. The 3.1-kb genomic DNA fragment of the LAI isolate was cloned behind the CMV immediate-early promoter in the expression vector pcDNA3. The resulting pcDNA3–Tat construct (Fig. 2) efficiently expresses Tat in a variety of cell types and, because of the presence of the SV40 origin of replication, produces high Tat levels in COS cells for detection by Western blotting (see below). The genomic HIV-1 sequences encode the exon-1 Tat form of 72 amino acids (aa) from unspliced transcripts, and splicing extends the open reading frame to 86 aa. Because the construct also encodes both the Rev response element (RRE) and the Rev protein, the unspliced transcript is expected to represent the predominant RNA form. This pcDNA3–Tat vector was used to express mutant Tat proteins with individual aa substitutions in the activation domain.

Four highly conserved aromatic aa in the cysteine/core domains were chosen as targets for mutagenesis (Fig. 1A). We substituted each of these residues with two alternative aromatic residues (conservative mutation) or alanine (nonconservative mutation). In addition, some mutants were generated on the basis of variation ob-

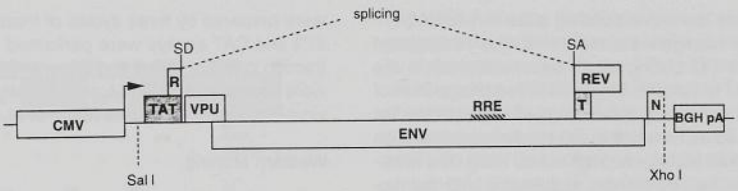



FIG. 2. Schematic of the Tat expression vector pcDNA3–Tat. Shown are the vector-derived Cytomegalovirus (CMV) immediate-early promoter (transcription start site is marked by an arrow) and the polyadenylation signal of the bovine growth hormone gene (BGHpA). *Sal*1 and *Xho*1 restriction sites denote the borders of the subgenomic HIV-1 LAI insert. Tat and T = first and second coding exons of Tat; R and Rev = first and second coding exons of Rev; N = truncated Nef open reading frame. The splicing signals (SD and SA) and the Rev response element (RRE) are indicated.

served at these positions in natural HIV and SIV isolates [e.g., Y26H, F32L, Y47H (Myers *et al.*, 1994)]. Thus, the tyrosine residue at position 26 was replaced by phenylalanine (Y26F), tryptophan (Y26W), alanine (Y26A), and histidine (Y26H). A similar set of mutants were generated for phenylalanine at position 32 and tryptophan at position 47. Please note that two of the Y47 mutants were generated in duplicate with alternative, synonymous codons (Y47H1/Y47H2 and Y47A1/Y47A2). The phenylalanine residue at position 38 was replaced by tryptophan (F38W).

Transient transfections in COS cells were performed to analyze the stability of the mutant Tat proteins. Cell lysates were prepared and separated by SDS–PAGE, blotted on nitrocellulose membranes, and stained with a Tat-specific monoclonal antibody (Fig. 3). The expression level of all mutant proteins was comparable to that of wild-type Tat. As expected, wild-type Tat and the Y26, F32, and F38 mutants were expressed predominantly in the unspliced exon-1 form (72 aa, 14 kDa). In contrast, all Y47 mutants except Y47H1 shift the expression toward the spliced exon 1 + 2 form (86 aa, 17 kDa). One particular feature of Tat codon 47 (UAU) is that it overlaps the start codon of the Rev open reading frame (AUG). Mutation of Tat codon 47 is therefore expected to affect Rev translation by changing either the initiation codon or the upstream Kozak consensus sequence (Kozak, 1989). The sequences around the Rev start codon of all Tat codon 47 variants are listed in Fig. 1B, and the effect on Rev translation is indicated. Splicing will be efficient in the absence of Rev synthesis, leading to increased synthesis of the 2-exon 86-aa Tat protein. Consistent with these predictions, all Y47 mutants except Y47H1 affect Rev translation. However, no gross differences in the steady-state Tat protein levels were measured. Because subsequent Tat *trans*-activation assays were performed in the SupT1 T-cell line, we tested expression levels for a subset of the Tat proteins in these cells (Fig. 4). Consistent with the results in COS cells, no major differences were measured in the intracellular protein levels, and the Y47W mutant with the Rev defect did not produce the exon 1 form of the Tat protein.

### A strict correlation between Tat activity and viral replication capacity

The activity of the Tat mutants was first tested in co-transfections with an LTR–CAT reporter plasmid in different cell lines. The results presented in Fig. 5 represent the mean values of three to six independent transfections, and the activity of the wild-type Tat protein was set at 100% for each cell type. In general, similar results were obtained for all four cell types tested. We therefore refer mainly to the results obtained in the T-cell line SupT1. The conservative mutants Y26F and Y26W, as well as the natural variant Y26H, demonstrated approximately full activity in SupT1 cells (79–103%). The Y26H mutant

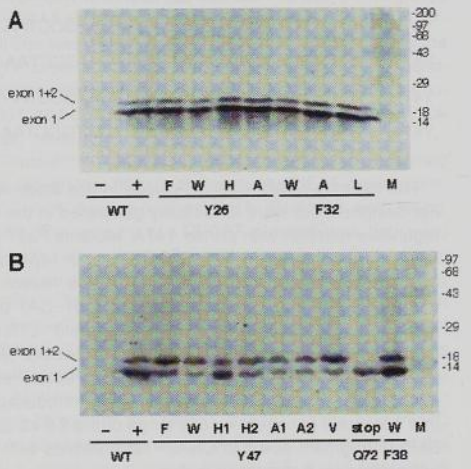



FIG. 3. Wild-type and mutant Tat proteins are stably expressed in COS cells. Western blot analysis of Tat protein expression in transiently transfected COS cells. The wild-type Tat (WT) is shown next to a mock-transfected sample (–). Lane M contains marker proteins (molecular mass in kilodaltons). The set of Tat mutants at positions Y26 and F32 in A was stained with Ab 2. The blot in B was probed with Ab 4 and contains samples of the Tat mutants at position Y47, the single F38W mutant, and a Q72stop variant with a premature stop codon to yield a Tat protein of 71 amino acids (Verhoef and Berkhout, in preparation).

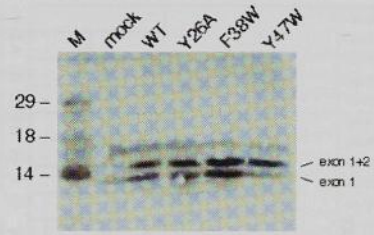



FIG. 4. Five million SupT1 T cells were electroporated with 50 μg of the individual Tat plasmids, and harvested on Day 2 post-transfection. The immunoblot was stained with mAb 4. See Fig. 3 for further details.

dropped to about 50% activity in the other cell types and the nonconservative Y26A mutant was severely defective with less than 10% activity. The one conservative mutant tested at position F32W retained considerable activity (74%). The natural variation F32L was also partially active (47%), but F32A was completely inactive (1%). The singular mutant at position F38W was partially active (42%). Similarly, all codon 47 mutants displayed reduced activity in SupT1 cells (10–47%), although the conservative mutant Y47F was slightly better than the other mutants, including Y47W, in the other cell types. In contrast to the other aromatic positions tested, Y47 replacement by alanine does not fully inactivate the protein.

In general, similar results were obtained with this set of Tat mutants in the SupT1 T-cell versus the non-T-cell lines HeLa and COS. However, the Tat defects scored in the latter cell type were generally less severe. For instance, all Y47 mutants were active in COS cells (77–129%), but significant defects were measured in HeLa (27–66%) and SupT1 (10–47%) cells. Furthermore, the most defective Tat mutants in SupT1 and HeLa cells (Y26A and F32A, less than 10% activity) show substantial activity in COS cells (18–22%). To test the activity of the mutant Tat proteins on a chromosomally integrated LTR–CAT reporter, we also transfected HLCD4-CAT cells. No significant differences were measured in comparison with the parental HeLa cells (Fig. 5).

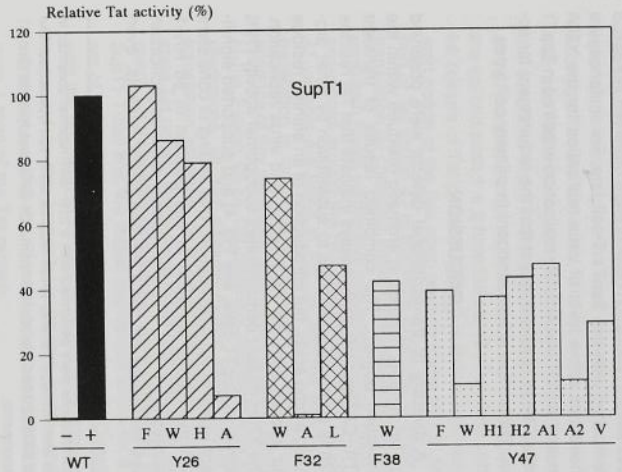
We next tested the complete set of Tat mutants in viral replication studies to determine the level of Tat activity required for virus replication. Furthermore, any discrepancies between the two assay systems may be indicative of nontranscriptional roles of Tat in the replication cycle. The SupT1 T cell was transfected with the individual proviral constructs and viral replication was monitored by measuring CA-p24 antigen production in the culture medium (Fig. 6). Compared with the wild-type virus, all mutants exhibited some decrease in replication capacity. No virus production was measured for Y26A, F32A, and all codon 47 mutants except Y47H1. We do not discuss these codon 47 mutants further because their replication defect is in part due to mutation of the Rev start codon (Fig. 1B, with the exception of mutant Y47H1). These results reveal a strict correlation between the Tat transcriptional activity as measured in transient LTR-CAT assays and the replication potential of the mutant viruses. The ranking order of the replication potential of these viruses is as follows, with the relative LTR-CAT activity in SupT1 cells shown within parentheses: wild type (100%) > Y26H (79%) > Y26F (103%) > Y26W (86%) ≫ F32W (74%), F32L (47%) > Y47H1 (37%) > F38W (42%) ≫ Y26A (7%), F32A (0%). The correlation coefficient for Tat activity and viral replication was measured on Day 5 post-transfection, excluding the Rev$^-$ viruses. There was a linear correlation between Tat activity and the log of virus production, with an $R^2$ value of 0.82 ($P < 0.0005$).

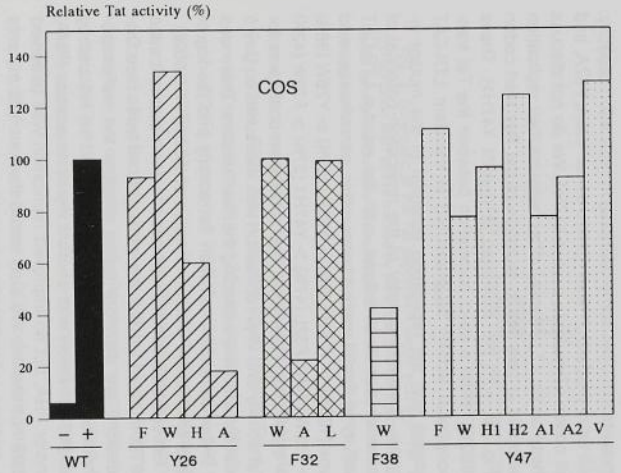
Several studies have reported considerable replication of Tat-mutated viruses in a variety of cell types (see Discussion). We therefore tested whether the replication-impaired mutants Y26A and F38W could be rescued by cellular activation with phorbol myristate acetate (PMA) and/or PHA, but no improved replication was observed (data not shown). We also tested the four Y26 mutants in primary cells. PBMCs were electroporated with the wild-type and mutant constructs and virus replication was monitored for up to 14 days (Fig. 7). In general, less severe replication defects were observed in PBMCs compared with the SupT1 T-cell line. For instance, we measured a low level of virus replication for the Y26A mutant, which is absolutely replication-impaired in SupT1 cells. These results suggest that the requirement for active Tat protein is less stringent in primary cell types.

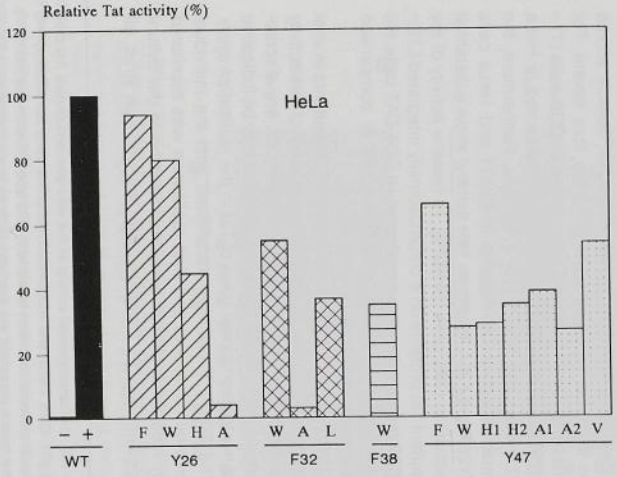
## DISCUSSION

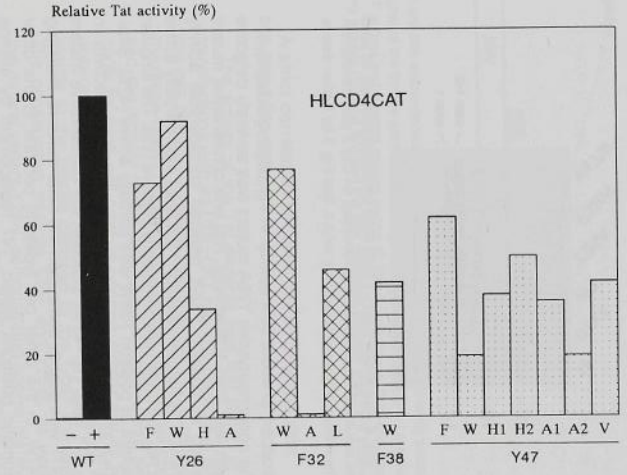
A diversity of transcriptional defects were observed for Tat variants mutated at aromatic residues within the activation domain. The combined results of transient LTR–CAT and infectivity assays indicate that Tat residue Y26 is relatively insensitive to substitution by other aromatic amino acids, but introduction of an alanine residue did abolish both the Tat function and virus replication. The Y26H mutant demonstrates suboptimal activity (79% activity in SupT1 cells, see Fig. 4) and supported significant levels of virus replication. This finding is consistent with the natural variation at this position in 56 HIV-1 isolates (51 Y, 4 H, 1 F). Significantly reduced replication was measured for the Y26F and Y26W mutants, even

FIG. 5. Transcriptional activity of the wild-type and mutant Tat proteins. Cotransfections were performed with a LTR–CAT reporter construct in SupT1, COS, HeLa, and HLCD4-CAT cells. The *trans*-activation activity obtained with wild-type Tat was set at 100% for every cell type tested. The actual promoter induction levels with wild-type Tat were approximately 100-fold (COS), 20-fold (HeLa), 10-fold (HLCD4-CAT), and 300-fold (SupT1). The results presented are averages of three to six independent transfection experiments.


Relative Tat activity (%)
SupT1
120
100
80
60
40
20
0
– +
F W H A
W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47


Relative Tat activity (%)
COS
140
120
100
80
60
40
20
0
– +
F W H A
W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47


Relative Tat activity (%)
HeLa
120
100
80
60
40
20
0
– +
F W H A
W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47


Relative Tat activity (%)
HLCD4CAT
120
100
80
60
40
20
0
– +
F W H A
W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47

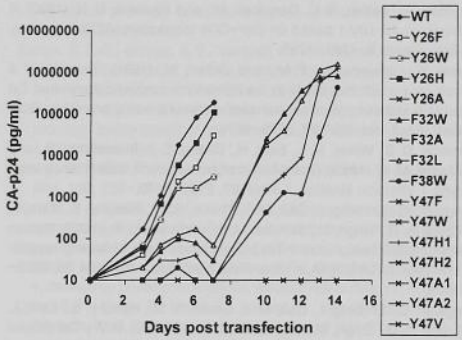



FIG. 6. Replication kinetics of Tat-mutated HIV-1 viruses. The SupT1 T-cell line was transfected with 1 μg of the wild-type and mutant constructs, and virus production was measured in the culture supernatant by CA-p24 ELISA several days post-transfection. The culture was split on Days 5 and 7 post-transfection, causing a slight drop in CA-p24 values. No replicating virus was recovered from transfections with mutants Y26A, F32A, and all Rev$^-$ codon 47 mutants; all these samples are marked with crosses.

though the Tat activity in transient transcription assays reached levels (103 and 86%) at least equal to that of the Y26H mutant. The reduced replication capacity of these two Tat mutants correlates with the natural variation at this position; only one natural HIV-1 isolate has a phenylalanine (F) at position 26 and no isolates have been reported with a tryptophan (W) at this position.

Significant Tat activity was retained on introduction of a conservative mutation at position 32 (F32W, 74% activity in SupT1 cells), but a rather severe effect on viral replication was measured. The nonconservative F32L mutation did partially reduce Tat activity (47%) and virus replication. Both amino acid substitutions are present in a significant number of the 56 natural HIV isolates (25 F, 20 Y, 7 L, 4 W). This result suggests that suboptimal Tat function is allowed *in vivo*. Alternatively, one cannot exclude the possibility that compensatory, second-site mutations are present within the Tat protein of these isolates. A conservative mutation at Tat position 38 (F38W) demonstrated reduced Tat activity (42%) and a significant delay in virus replication. Consistent with this result, residue F38 is absolutely conserved in natural HIV-1 isolates. We measured considerable Tat activity for all Y47 mutants, but no replicating virus was recovered with the exception of the Y47H1 mutant. This discrepancy is explained by the fact that mutations in Tat codon 47 also affect the overlapping start codon of the Rev gene. In fact, Y47H1 is the only mutant at position 47 that maintains the AUG start codon (Fig. 1B). This additional sequence requirement does explain the nearly absolute conservation of this Tat residue in 56 natural isolates (55 Y, 1 H).

The set of Tat activation domain mutants allowed us to accurately determine the level of Tat activity required for virus replication. We found that a small reduction in Tat activity does already affect viral infectivity (e.g., F32L and F38W, 47 and 42% Tat activity in SupT1 cells, respectively), and no viral replication was measured in the SupT1 T-cell line for Tat mutants with less than 10% activity (e.g., Y26A). In general, no gross discrepancies were apparent between the transient transcription and viral replication assays, but the mutants Y26F, Y26W, and F32W exhibited a more severe replication defect than was expected on the basis of their transcriptional activity in SupT1 cells. These differences may be indicative of an additional role of the Tat protein in virus replication (Huang *et al.*, 1994), but further experimentation is required to elaborate on this point.

We also tested the Tat-mutated viruses for their ability to replicate in primary cell types. Replication defects were also apparent in PBMCs, albeit less dramatic than in the transformed T-cell line SupT1. Cellular activation by PMA and/or PHA did not change the replication potential of Tat-mutated viruses. Several studies reported considerable replication of Tat-mutant viruses in a variety of cell types (Dimitrov *et al.*, 1993; Chang and Zhang, 1995; Zhu *et al.*, 1996; Luznik *et al.*, 1995; Duan *et al.*, 1994). Thus, it appears that HIV-1 replication is dependent primarily on the Tat function in transformed T-cell lines, which may resemble mature, peripheral T cells in HIV-infected individuals. The observation that Tat is most critical in transformed T-cell lines is diametrical to the situation described for the so-called accessory HIV-1 gene products (Nef, Vif, VpR, VpU), which are required predominantly in primary cell types (reviewed by Subramanian and Cohen, 1994). The relative unimportance of Tat in certain cell types may explain the abundance of Tat-mutated proviruses in clinical samples (Meyerhans *et al.*, 1989; Sabino *et al.*, 1994).

Previous studies with Tat mutants containing either truncations or substitutions in the basic domain identified a *trans*-dominant negative phenotype (Pearson *et al.*, 1990; Modesti *et al.*, 1991; Orsini and Debouck, 1996).

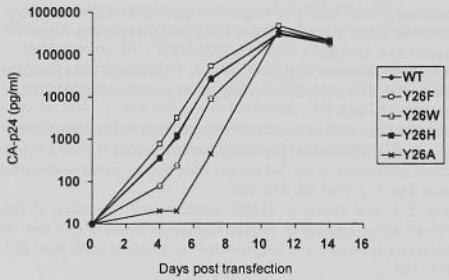



FIG. 7. Replication kinetics of Y26-mutated HIV-1 variants in primary cells. PBMCs were transfected with 2 μg of the wild-type and mutant constructs and virus production was measured in the culture supernatant by CA-p24 ELISA several days post-transfection.

Such proteins have low intrinsic activity, but are powerfully capable of inhibiting the wild-type Tat protein function in *trans*. We therefore tested all Tat mutants with less than 50% transcriptional activity in cotransfections with equimolar amounts of wild-type Tat, but none of the mutants displayed a *trans*-dominant negative phenotype (results not shown).

The Tat mutants provide a nice set of reagents with a diverse activity spectrum that can be used for further biochemical analysis of Tat-associated cellular cofactors. In addition, characterization of additional Tat-mutated viruses is expected to reveal more information about the structure and function of the HIV-1 Tat protein. In particular, it will be of interest to select second-site mutations within the Tat gene that are able to restore the function of inactive Tat mutants. Compared with mutations that merely block function, these gain-of-function mutations are expected to be easier to interpret structurally. Such a genetic analysis of revertant viruses would allow us to identify intramolecular contacts within the Tat protein.

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# Chapter 6

Koen Verhoef and Ben Berkhout:

*Journal of Virology 73 (1999), in press*

# A second-site mutation that restores replication of a Tat-defective Human Immunodeficiency Virus.

Abstract

We previously constructed a large set of mutants of the HIV-1 regulatory protein Tat with conservative amino acid substitutions in the activation domain. These Tat variants were analyzed in the context of the infectious virus and several mutants were found defective for replication. In an attempt to obtain second-site suppressor mutations that could provide information on the Tat protein structure, some of the replication-impaired viruses were used as a parent for the isolation of revertant viruses with improved replication capacity. Sequence analysis of revertant viruses frequently revealed changes within the *tat* gene, most often first-site reversions either to the wild-type amino acid or to related amino acids that restore, at least partially, the Tat function and virus replication. Out of 30 revertant cultures, we identified only one second-site suppressor mutation. The inactive Y26A mutant yielded the second-site suppressor mutation Y47N that partially restored transactivation activity and virus replication. Surprisingly, when the suppressor mutation was introduced in the wild-type Tat background, it also improved the transactivation function of this protein about 2-fold. We conclude that the gain of function measured for the Y47N change is not specific for the Y26A mutant, arguing against a direct interaction of Tat amino acids 26 and 47 in the three-dimensional fold of this protein. Other revertant viruses did not contain any additional Tat changes and some viruses revealed putative second-site Tat mutations that did not significantly improve Tat function and virus replication. We reason that these mutations were introduced by chance through founder effects or by linkage to suppressor mutations elsewhere in the virus genome. In conclusion, the forced evolution of mutant HIV-1 genomes, which is an efficient approach for the analysis of RNA regulatory motifs, seems less suited for the analysis of the structure of this small transcription factor, although protein variants with interesting properties can be generated.

## Introduction

X-ray crystallography is a powerful tool for the study of protein structure and function. However, the use of this method is limited to proteins that crystallize. The HIV-1 Tat protein is essential for virus replication and is a unique transcriptional transactivator protein. Tat is recruited to the HIV-1 LTR promoter by binding to an RNA hairpin structure termed TAR, which is formed at the 5' end of viral messenger RNA (7,13). Tat is encoded on two exons and 86-101 amino acids in length, depending on the viral isolate. The first 72 amino acids encoded by the first exon are sufficient for the transactivation function (17,22,28,33,39). In addition to its essential role in LTR transcription, Tat has been suggested to be involved in other steps of the virus life cycle. Tat has been reported to stimulate the process of reverse transcription (16) and to increase the translational efficiency of HIV-1 mRNAs (8,34,36,44). Despite intensive research for over 10 years, this protein of biological and medical importance has resisted attempts to solve its structure by X-ray crystallography. Furthermore, only limited resolution was obtained by NMR studies (3,14,26).

In this study, we assess the potential of a genetic approach termed 'forced evolution' for the analysis of the Tat protein structure and function. The systematic analysis of revertant virus genomes is particularly useful for the dissection of sequence and structural determinants of RNA signals that control a variety of steps in the viral replication cycle (6,21,29,30). In addition, intragenic suppressor mutations in revertant HIV-1 viruses have been described for the Envelope glycoprotein (43) and the Integrase enzyme (37). The *tat* gene of an infectious HIV-1 genome was mutated to introduce single amino acid changes within the cysteine-rich transactivation domain. We identified several Tat-mutated viruses that exhibit a severe replication defect in T cell lines and primary cells (41). In this study, some of the replication-impaired viruses were used as starting material for long-term cultures to allow the generation of faster replicating revertant viruses. Such virus revertants may have compensated for the introduced mutation by second-site changes elsewhere in the protein, and putative interaction sites can be revealed by this genetic technique.

Two replication-impaired HIV-1 variants with a severely inactivated Tat protein (mutants Y26A and F32A) and two poorly replicating viruses with a partially active Tat protein (F38W and Y47H) were cultured for a prolonged period in multiple, independent evolution experiments. We were able to generate fast replicating revertant viruses in 30 cultures, of which 21 contained an additional, non-silent mutation within the *tat* gene. Of these 21 revertants, 11 first-site revertants were obtained that had replaced the mutant amino acid, either by true first-site reversion to the

wild-type amino acid, or by mutation to a residue different from that observed in the wild-type Tat protein. Several putative second-site suppressor mutations were observed in Tat, but only one demonstrated improved Tat activity in transient transfection assays.

The Y26A Tat mutant regained partial activity by inclusion of the Y47N change, and a concomitant increase in virus replication was measured. Surprisingly, when this second-site change was introduced as an individual mutation in the wild-type Tat protein, it improved the activity of this protein to merely 200%. Thus, the Y47N mutation represents a more general manner to make a more active Tat protein, which argues against a specific amino acid contact between Tat position 26 and 47. We will discuss potential reasons for the absence of a 200% active Tat protein in natural HIV/SIV isolates. The other second-site mutations did not improve the Tat transactivation function, but could have restored a putative additional function of Tat in the viral life cycle. We tested this for some of the revertants in virus replication studies, but none of the revertants demonstrated enhanced fitness compared with the corresponding mutants. We propose that these second-site mutations represent either natural Tat variation or changes that were linked to mutations elsewhere in the HIV-1 genome that did contribute to the reversion event. These results indicate that the systematic analysis of mutant-revertant viruses is not a particularly efficient method to gain insight into the structure of this small, regulatory viral protein, although this method yielded some Tat variants with intriguing properties.

## Materials and Methods

**Cell culture, virus infections and DNA transfection.** The construction of the Tat-mutated HIV-1 LAI proviral clones was described previously (41). The Y47H mutant that was used in this study corresponds to the Y47H2 mutant (codon CAC) described in that study. SupT1 and C8166 T cells were cultured in RPMI medium, supplemented with 10% Fetal Calf Serum (FCS), 100U/ml penicilline and 100 µg/ml streptomycine and transfected by means of electroporation (11). For the selection of revertant viruses in forced evolution experiments, we used 30 µg of mutant pLAI plasmid to transfect 5 x $10^6$ SupT1 or C8166 cells. One day after transfection, the cells were split 1 in 6 and divided over a 6-well culture plate to obtain independent reversion events. These cells were cultured for up to 160 days after transfection, splitting the culture 1 in 10 every four days. Once virus spread was evident by the appearance of syncytia, we passaged the virus-containing culture supernatant at the peak of infection onto fresh T cells. We initially used 100 µl of cell-free supernatant to infect a 5 ml T cell culture, but this amount was gradually

reduced to 0.1 µl. At regular intervals, infected cells were taken from the culture and frozen for subsequent analysis of the proviral DNA.

Transient transfection of SupT1 cells for CAT-assays or viral replication studies has been described previously (41). C33A cells were grown in DMEM medium with supplements as described (11) and transfected using the calcium phosphate precipitation method. Briefly, cells were grown to 60% confluency in 60 mm culture dishes. Unless indicated otherwise, we used 1 µg of LTR-CAT reporter plasmid and 100 ng of pTat in transient transfections. The total amount of DNA in the transfection was adjusted to 6 µg with pcDNA3 carrier plasmid in 132 µl $H_2O$, mixed with 150 µl 50 mM Hepes (pH 7.1)-250 mM NaCl-1.5 mM $Na_2HPO_4$ and 18 µl 2 M $CaCl_2$, incubated at room temperature for 20 min, and added to the culture medium (4 ml). The cells were washed the next day and fresh culture medium was added.

**CAT assays and CA-p24 ELISA.** Transiently transfected cells were collected by trypsinization (adherent cell types) or centrifugation (non-adherent cell types) at three days posttransfection. CAT assays were performed on whole cell lysates using the phase-extraction protocol (32). TheCA-p24 level in cell-free supernatant from virus cultures was determined by antigen capture ELISA (2).

**Proviral DNA analysis and cloning of revertant sequences.** The Tat expression vector pTat used in this study is a derivative of pcDNA3-Tat (41). A 61 bp *Hind*III-*Hind*III fragment in the polylinker of pcDNA3-Tat was deleted to remove an *Asp*718I site for subsequent cloning purposes. This modified Tat-expression vector pTat was used throughout this study. Total cellular DNA from infected cells was isolated as described previously (11). Proviral *tat* sequences were amplified from total cellular DNA by a standard 35-cycle PCR reaction, using sense primer KV1 (5' CCATCGATACCGTCGACATAGCAGAATAGG 3') and the antisense primer WS3 (5' TAGAATTCTTGATCCCATAAACTGATTA 3'). The 797 bp product was cleaved at the 5' terminus with *Cla*I (recognition site in primer underlined) and with *Asp*718I downstream of the first Tat coding exon and cloned into pTat, thus replacing the wild-type *tat* gene. Several pTat clones derived from an individual culture were sequenced to determine the sequence variation in the virus population. Sequence analysis was performed with a T7 DYEnamic Direct cycle sequencing kit (Amersham) on an automated DNA sequencer (ABI). Several revertant Tat sequences were subsequently cloned from the pTat vector into the pLAI infectious molecular clone by exchange of a 2.6 kb *Sal*I-*Bam*HI fragment. The Y47N mutation was introduced into wild-type and F32A pTat by PCR mutagenesis (25) using mutagenic primer Y47N: 5' CTTCT-

TCCTGCCATTGGAGATGCCTAA 3' (mismatching nucleotide underlined). Cloning of Y47N into the pLAI plasmid was performed as described above. All constructs were verified by sequence analysis.

**RNA isolation and primer extension assay.** As an internal control for primer extension analysis, we constructed a modified LTR-CAT reporter plasmid by filling in the *Hind*III site that fuses the HIV-1 LTR promoter to the *cat* gene, thus creating transcripts with a 4 nucleotide insertion (LTR-CAT+4). Transfections of C33A cells for primer extension analysis contained equal amounts of LTR-CAT and LTR-CAT+4. Cells were harvested 2 days after transfection and total RNA was isolated by the hot phenol method (4), ethanol precipitated and dissolved in 20 µl of TE. 1 µl RNA was used for primer extension analysis as described previously (10) with some minor modifications. A new primer, complementary to the 5' end of the *cat* open reading frame, was used (Sp6CATAUGrev: 5' CGATTTAGGTGACACTATAGCTCCATTTTAGCTTCCTT-AGC 3'). Reverse transcription was performed at 42 °C, and the reaction was stopped by addition of 1 µl 0.5 M EDTA, denatured in the presence of formamide and analyzed on a 6% polyacrylamide sequencing gel. The cDNA products were quantitated on a Phosphorimager (Molecular Dynamics).

**Western blotting.** Subconfluent COS cells (60mm dish) were transfected with 10 µg of the Tat expression vectors by the DEAE-dextran method. The cells were lysed in Laemmli-buffer two days after transfection and the proteins were resolved on a 20% SDS-PAA gel. Mouse monoclonal anti-Tat antibody #4 was used to detect Tat protein in a Western blot analysis as described previously (40).

Table 1: Overview of Tat mutants and revertants

| Tat mutant (activity) | Culture number | Evolution time (days) | Tat amino acid change [1] | Tat codon change | Tat activity [2] | Improved replication [3] |
|---|---|---|---|---|---|---|
| Y26A | K8 | 45 | A26W (4/4) | GCC->UGG | 86% | Yes |
| UAU->GCC | K9 | 161 | Y47N (6/6) | UAU->AAU | 23% | Yes |
| (7%) | Z1-2 | 58 | - | - | - | - |
| | Z1-3 | 58 | - | - | - | - |
| | Z1-5 | 62 | A26Y (3/3) | GCC->UAU | 100% | Yes |
| | A5 | 77 | - | - | - | - |
| | Y-2 | 104 | - | - | - | - |
| | Y-5 | 137 | A26F (2/2) | GCC->UUC | 103% | Yes |
| | 6.5 | 47 | K50R (1/1) | AAG->AGG | 5% | No |
| F32A | V1 [4] | 98 | K29R (10/10) | AAG->AGG | 5% | No |
| UUU->GCC | Z4-1 | 58 | A32V (4/4) | GCC->GUC | 15% | No |
| (4%) | Z4-2 | 58 | A32V (4/4) | GCC->GUC | 15% | No |
| | Z4-3 | 62 | K29R (2/5) | AAG->AGG | 5% | No |
| | Z4-4 | 55 | - | - | - | - |
| | Z4-5 | 62 | - | - | - | - |
| | Z4-6 | 62 | A32V (1/2) | GCC->GUC | 15% | No |
| | A1 | 104 | Q54R (4/4) | CAG->CGG | 4% | No |
| | A2 | 101 | A32V (3/3) | GCC->GUC | 15% | No |
| | A3 | 21 | A32F (3/4) | GCC->UUC | 100% | Yes |
| | | 49 | A32F (5/5) | GCC->UUC | | |
| | A4 | 77 | - | - | - | - |
| | A5 | 77 | - | - | - | - |
| | A6 | 21 | A32F (3/4) | GCC->UUU | 100% | Yes |
| | | 49 | A32F (5/5) | GCC->UUU | | |
| | A1 NS | 68 | S70P (2/4) | UCA->CCA | 6% | n.d. |
| | A2 NS | 68 | C27Y (1/2) | UGU->UAU | 1% | n.d. |
| | A3 NS | 68 | - | - | - | - |
| | A4 NS | 68 | A32V (3/4) | GCC->GUC | 15% | No |
| | A5 NS | 68 | A32V (4/4) | GCC->GUC | 15% | No |
| | A6 NS | 68 | K29R (2/3) | AAG->AGG | 5% | No |

| | | | | | | |
|---|---|---|---|---|---|---|
| F38W UUC->UGG (42%) | Q1.2 | 55 | K29R (12/12) | AAG->AGG | 46% | n.d. |
| Y47H UAU->CAC (43%) | V4 [4] | 128 | Q17K (2/2) | CAG->AAG | 35% | n.d. |

[1] first site mutations are shaded (in brackets the ratio of clones containing the Tat mutation)
[2] measured in LTR-CAT + pTat cotransfection of SupT1 cells; wild-type = 100%
[3] revertant *tat* gene reconstructed in the wild-type HIV-1 background
[4] evolution in the C8166 T cell line
n.d. = not determined

## Results

**Tat-mutated HIV-1 and the selection of revertant viruses.** We previously constructed a set of Tat mutants in the HIV-1 LAI isolate (41). Substitution of the aromatic amino acids tyrosine at position 26 and phenylalanine at position 32 by alanine (mutants Y26A and F32A) resulted in replication-impaired viruses. Other mutants include a tryptophane for phenylalanine substitution at position 38 (F38W) that is reduced in Tat activity and virus growth and mutant Y47H. Tat codon 47 (UAU) is special in that it overlaps the Rev translation initiation codon by two nucleotides (...UAUG...). Although the Y47H Tat protein is partially active, virus replication is abrogated because the Rev translation initiation codon is disrupted (...CACG...)(41). This set of four Tat mutants was used in this study to select for revertant viruses in prolonged cultures. Some other Tat mutants that are defective in replication were also subjected to forced evolution, but we failed to obtain revertant viruses. In particular, other Tat codon 47 mutants with the Rev- genotype could not be reactivated except for a single Y47H culture. Reversion of these virus mutants may be particularly difficult because both the Tat activity and Rev expression need to be restored. In the design of the other Tat mutants, the codon was changed such that it would be relatively difficult for the mutant virus to revert to the wild-type amino acid. For instance, we used the alanine codon GCC to substitute for the phenylalanine codon UUU at position 32. Reversion to the wild-type codon requires mutation of all three nucleotides, whereas only two changes would be required if the alanine codon GCU was used. In other words, we tried to optimize the chances of selecting for Tat revertants with second-site mutations by restricting the ability to generate wild-type revertants.

Forced evolution was initiated by massive transfection of the mutant molecular clones into the SupT1 T cell line. The cultures were maintained to allow virus spread until any replication-competent variant could expand to a significant portion of the cells, as indicated by CA-p24

production in the culture supernatant and the appearance of virus-induced multinucleated cells (syncytia). Once virus production was apparent, we passaged the cell-free supernatant onto uninfected SupT1 cells, initially with a large inoculum (up to 100 µl supernatant), but this amount was gradually decreased (e.g. 0.1 µl to infect $10^6$ T cells in a 5 ml culture). To determine the range of mechanisms by which these mutant viruses could restore replication, we attempted to recover revertant viruses in multiple, independent cultures of the Y26A mutant (32 cultures), the F32A mutant (21 cultures) and the Y47H mutant (8 cultures). The cultures in which we succeeded to select for a fast replicating HIV-1 variant are listed in Table 1. Indicated is the original Tat mutation, the culture number and the time that this evolution experiment was maintained (in days posttransfection). We obtained fast replicating virus in a significant number of the evolution experiments with mutant Y26A (9 cultures) and in almost all F32A infections (19 cultures). Infections started with the Y47H mutant did yield only one positive virus culture. We also analyzed viruses in one long-term culture of the poorly replicating, but not completely defective F38W mutant.

**Sequence analysis of revertant viruses.** The SupT1 cells of cultures containing a replicating virus variant were harvested at the day indicated in Table 1 and total cellular DNA was isolated. A portion of the integrated HIV-1 proviral genome including the first Tat coding exon was amplified by PCR and cloned into the pTat expression plasmid for sequence analysis and transient expression of the variant Tat protein. At least two independent clones were sequenced for each revertant to recognize mutations that may have been introduced during PCR amplification of the proviral genome. In some cultures, Tat sequences were analyzed at multiple times during evolution (e.g. F32A culture A3, days 21 and 49 posttransfection, Table 1). The amino acid changes observed within the first Tat coding exon and the corresponding codon changes are listed in Table 1. In general, it is obvious that some of the revertant viruses did not acquire any mutations in Tat, and these viruses may have improved their replication fitness by other means (see discussion). We will focus on those revertants that do contain an additional amino acid substitution in Tat, either at the site of mutation (first-site) or at other positions (second-sites).

The Y26A mutant. We analyzed all nine revertant cultures of the total of 32 infections that were started with the Y26A mutant. Five cultures acquired a non-silent mutation in the first Tat coding exon, three at the first-site (marked in grey in Table 1) and two at a second-site. Thus, two putative second-site Tat revertants were obtained, Y26A-Y47N and Y26A-K50R, and both will be analyzed in further detail lateron.

In the three first-site Tat revertants, the mutated A26 residue was changed either back to the wild-type amino acid (tyrosine) or to other aromatic residues (phenylalanine and tryptophane). In fact, the latter two Tat variants were tested previously as part of a large mutational study (41). Efficient LTR-CAT transactivation was measured for the 26F and 26W variants (103% and 86% of the wild-type level, respectively). Furthermore, efficient virus replication was demonstrated for these two variants, thus confirming that these changes cause the reversion phenotype. These functional Tat data are included in Table 1. It is interesting that relatively difficult mutations were used to change the mutant alanine codon GCC into the codons for these aromatic residues, requiring three transversions (UGG, tryptophane), one transversion and one transition (UUC, phenylalanine), or two transversions and one transition for the wild-type revertant (UAU, tyrosine). Obviously, other possible codons could be generated by a more simple 1-nt substitution, including codons for threonine, serine, proline, aspartic acid, valine, and glycine. The absence of such non-aromatic amino acids in the revertant cultures suggests that these residues do not support Tat function and viral replication. Consistent with this result, non-aromatic residues are not observed at position 26 in the Tat protein of natural HIV-1 isolates, with the exception of histidine that, like aromatic residues, has a large ring structure (Table 2). These combined results demonstrate the importance of an aromatic side chain at position 26 of the Tat protein.

The F32A mutant. It was relatively easy to improve the fitness of the replication-impaired F32A mutant. We observed 19 reversions in a total of 21 cultures, and the corresponding viruses were analyzed. Five revertants did not show any amino acid change in the Tat protein. Of the remaining 14 cultures, 8 contained a first-site change (marked in grey, Table 1) and a putative second-site change was detected in 6 cultures: K29R (observed 3x), C27Y, Q54R, and S70P (each observed 1x). Some of these changes were not present in all of the clones that were sequenced per reversion event. For instance, C27Y was present in only 1 of the 2 clones, and S70P was observed in 2 of the 4 sequences (Table 1). Because Tat changes that determine the reversion phenotype are expected to become fixated in the virus population, these changes represent less likely candidates for a mutation that triggered the reversion. In contrast, the K29R mutation in culture V1 became fully fixated (10 out of 10 sequences), and this mutation also appeared in two other independent evolution experiments (cultures Z4-3 and A6 NS), suggesting that this Tat variation represents a true revertant. The putative F32A-K29R and F32A-Q54R second-site revertants were chosen for further analysis (see below).

Of the first-site changes of mutant F32A, we observed 2 mutations back to the wild-type residue phenylalanine and 6 mutations to valine. The two wild-type revertants were generated by different codon changes (from GCC to UUC and UUU), which underscores the independent

nature of these evolution experiments. The fact that the wild-type F32 revertant was obtained twice, and no tryptophane or tyrosine variants were selected at this position may indicate that, in contrast to position 26, other aromatic residues cannot replace F32. Indeed, the 32W variant was constructed previously and demonstrated reduced Tat activity (74%), coinciding with a substantial loss of virus fitness (41).

It was surprising that valine was observed 6 times as an alternative amino acid at position 32 because this residue is not present in natural isolates (Table 2). Frequent observation of the valine revertant may be caused in part by the relative ease of the corresponding nucleotide change (1-nt transition from GCC to GUC). Nevertheless, it is obvious that valine should at least partially restore Tat activity and virus replication. Indeed, we measured improved transcriptional activity for the 32V revertant compared with the 32A mutant in transient LTR-CAT assays (15% and 4% of the wild-type Tat activity, respectively, see Table 1). Introduction of the 32V mutation into pLAI demonstrated that this variation slightly improved virus replication (Fig 3C). We measured previously that the 15% Tat activity is below the threshold for efficient virus replication (41).

Table 2. Natural amino acid variation at Tat positions discussed in this study[1]

| Tat mutation (position) | amino acid occurence[2] |
|---|---|
| 26 | 51 Y, 4 H, 1 F |
| 32 | 25 F, 20 Y, 7 L, 4 W |
| 38 | 56 F |
| 47 | 56 Y, 1 H |

| Tat second-site mutation (position) | amino acid occurence[2] |
|---|---|
| 17 | 52 Q, 2 K, 1 T |
| 27 | 55 C, 1 G |
| 29 | 40 K, 7 R, 3 V, 2 I 1 L, 1 A, 1 C, 1 Q |
| 50 | 57 K |
| 54 | 48 Q, 3 R, 2 P |
| 70 | 31 S, 26 Pro |

[1] Survey of 57 HIV-1 isolates (26)
[2] Totals may deviate from 57 due to a number of incomplete Tat sequences (26)

The F38W and Y47H mutants. The single revertant culture of both the F38W and Y47H mutants was analyzed. The F38W virus maintained the original mutation and acquired a second-site mutation at position 29. This K29R mutation, which was also observed repeatedly in the context of the F32A mutant (see above), was present in all 12 clones that were sequenced, indicating that this amino acid substitution was fixated in the virus population. The Y47H Tat/Rev- mutant acquired the Q17K mutation as a putative second-site Tat adaptation, but this mutation does not repair the Rev start codon.

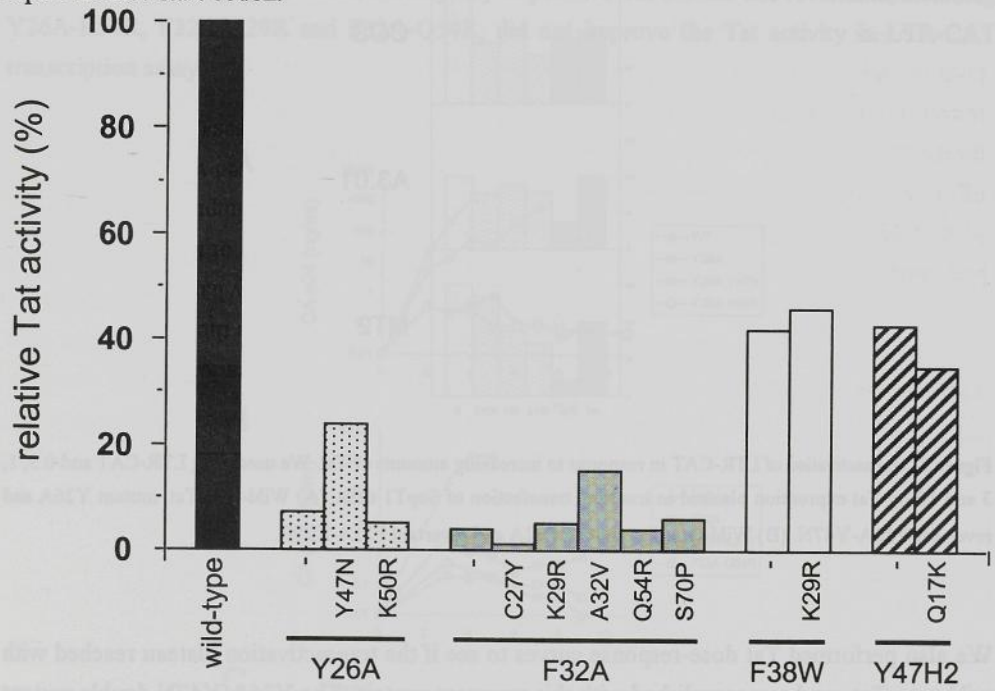


**Figure 1.** Transcriptional activity of mutant Tat proteins and putative revertants. Co-transfections were performed with the LTR-CAT reporter and the indicated Tat variants in the SupT1 T cell line. The transactivation activity obtained with wild-type was set at 100%. Results represent the average of two to eight transfection experiments.

**Functional tests of second-site Tat mutations.** Several putative second-site changes were observed in the Tat protein of revertant viruses. To test which Tat changes represent true second-site suppressor mutations, we analyzed them for recovery of Tat activity in transient LTR-CAT transfection assays. In Fig 1 we summarize the data of several independent transfection experiments. Wild-type Tat activity was set at 100% (corresponding to approximately 160-fold

induction of LTR promoter activity) and the mutant and revertant Tat proteins were tested in parallel for activation of HIV-1 gene expression. Mutant Y26A acquired two independent second-site mutations, Y47N and K50R. Whereas the K50R mutation did not improve the Tat activity, the Y47N mutation increased the activity of the Y26A mutant from 7 to 23% (Fig 1).

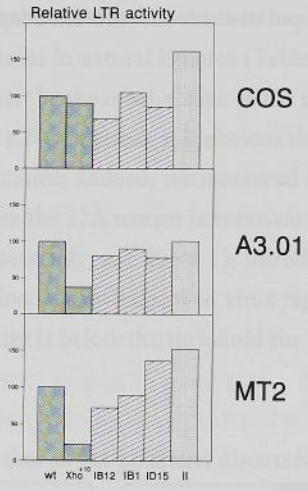


**Figure 2.** Transactivation of LTR-CAT in response to increasing amounts of Tat. We used 1 μg LTR-CAT and 0.3, 1, 3 and 10 μg Tat expression plasmid in transient transfection of SupT1 cells. (A) Wild-type Tat, mutant Y26A and revertant Y26A-Y47N. (B) Wild-type Tat, mutant F32A and revertant F32A-K29R.

We also performed Tat dose-response curves to see if the transactivation plateau reached with wild-type Tat can be accomplished with this revertant protein. The Y26A-Y47N double mutant transactivated the HIV-1 promoter to levels exceeding the 23% activity that was measured in the linear range of transactivation by the wild-type Tat protein (Fig 2B). In fact, at even higher Tat levels we measured transactivation levels that approximated the wild-type transactivation plateau (not shown). Thus, Y26A-Y47N represents a true second-site Tat revertant. As a final test for the reversion phenotype, we inserted the Y26A-Y47N genotype in the pLAI molecular clone. Note that the AAU codon for asparagine at position 47 does not disrupt the overlapping AUG start codon of the *rev* gene. Indeed, Y47N was able to rescue the replication defect caused by the Y26A mutation (Fig 3A). Thus, Y26A-Y47N is a *bona fide* second-site Tat revertant that partially restores the transactivation function of Tat, leading to a concomitant increase in virus fitness.

Second-site mutations that were observed in the F32A virus (C27Y, K29R, Q54R and S70P) did not improve the level of Tat-mediated LTR-CAT expression (Fig 1). This result was somewhat unexpected because the K29R mutation was selected in three independent reversions of the F32A mutant. We also tested Tat activity at varying levels of this transactivator protein, but no gain of function was apparent (Fig 2B). Likewise, the second-site changes observed in the F38W and Y47H viruses (F38W-K29R and Y47H-Q17K) did not improve the transactivation capacity of the mutant Tat proteins (Fig 1). Thus, the majority of putative Tat second-site revertants, including Y26A-K50R, F32A-K29R and F32A-Q54R, did not improve the Tat activity in LTR-CAT transcription assays.

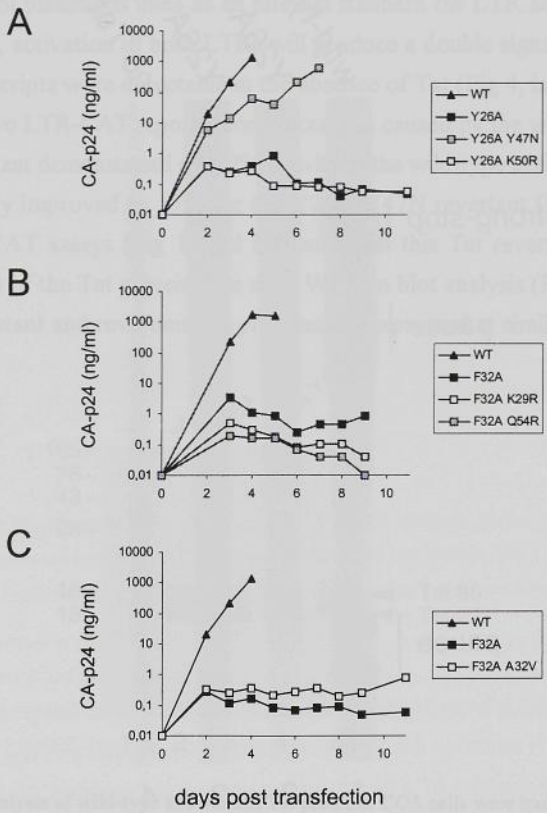


**Figure 3.** Replication kinetics of Tat-mutated HIV-1 viruses and revertants thereof. The SupT1 cell line was transfected with 5 μg of the wild-type, mutant or revertant pLAI constructs, and virus production was measured by CA-p24 ELISA at several times posttransfection. A slight drop in CA-p24 values is observed in some experiments due to dilution of the culture in order to sustain cell viability and virus replication.

It remains possible that these second-site Tat mutations rescue virus replication through an effect on another function that Tat may have in the HIV-1 replication cycle, e.g. in the process of reverse transcription (16,18). Thus, some of the adaptive changes within Tat may have been selected to improve such a non-transcriptional function. To critically test this, some of the yet unexplained second-site Tat mutations were introduced into HIV-1 LAI to screen for improved replication in comparison with the original mutant. However, we did not observe increased virus replication for the Y26A-K50R virus (Fig 3A), and the F32A-K29R and F32A-Q54R viruses (Fig 3B), ruling out a role of these second-site changes in the reversion event.

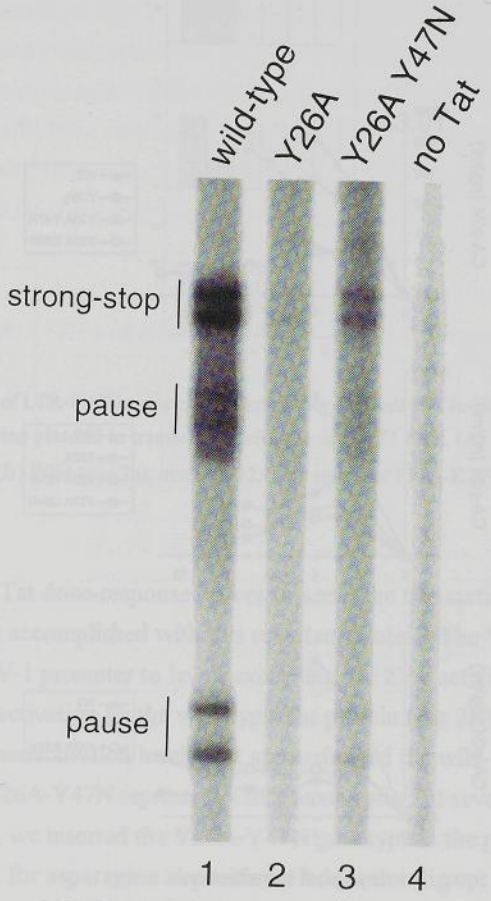


**Figure 4.** Primer extension analysis of Tat-induced transcripts. RNA was isolated from C33A cells that were transfected with two different LTR-CAT reporter constructs (see materials and methods section) and wild-type Tat (lane 1), mutant Y26A (lane 2), revertant Y26A-Y47N (lane 3) or the control vector (lane 4). Indicated are the full-length cDNA products and pause products that are due to stalling of the RT enzyme.

**The Y47N suppressor mutation functions at the transcriptional level.** The LTR-CAT transactivation results obtained with the Y26A-Y47N double mutant do not discriminate between an effect of this second-site Tat revertant at the level of transcription or translation. To test the transcription function of the Tat revertant, we performed direct RNA analyses on extracts of cells that were transiently transfected with the LTR-CAT reporter construct and a Tat expression

vector. Total RNA was isolated two days after transfection and used in primer extension assays (Fig 4). In this assay, we used equimolar amounts of the wild-type LTR-CAT construct and a modified LTR-CAT vector with a neutral 4-nt insertion at position +77 of the HIV-CAT fusion transcript. This control plasmid is used as an internal standard for LTR activity in case mutant LTRs are tested. Here, activation of both LTRs will produce a double signal in primer extension assays. No RNA transcripts were detectable in the absence of Tat (Fig 4, lane 4), but a dramatic up-regulation of the two LTR-CAT reporter constructs was caused by the wild-type protein (lane 1). The Y26A Tat mutant demonstrated only 7% activity of the wild-type activity (lane 2), but this value was significantly improved to 17% for the Y26A-Y47N revertant (lane 3). This result is consistent with the CAT assays (Fig 1) and indicates that this Tat revertant has restored the transcriptional function of the Tat protein. We used Western blot analysis (Fig 5) to demonstrate that the wild-type, mutant and revertant Tat proteins are expressed at similar levels.

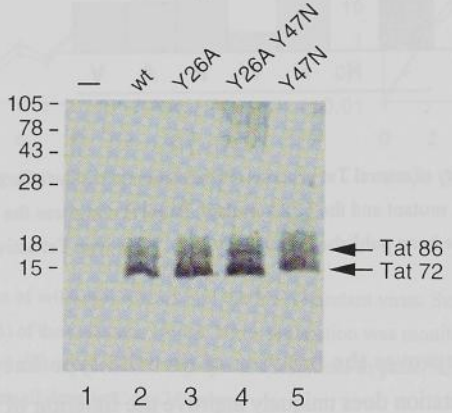


**Figure 5.** Western blot analysis of wild-type and variant Tat proteins. COS cells were transfected with 10 μg of the indicated Tat-expression vectors (lanes 2-5). Lane 1 contains a mock-transfected COS cell sample. Total cell extracts were prepared at two days posttransfection and analyzed on a Western blot that was stained with Tat monoclonal anitbody. The two Tat forms of 72 and 86 amino acids are indicated (41). The positions of the molecular mass marker proteins are indicated on the left.

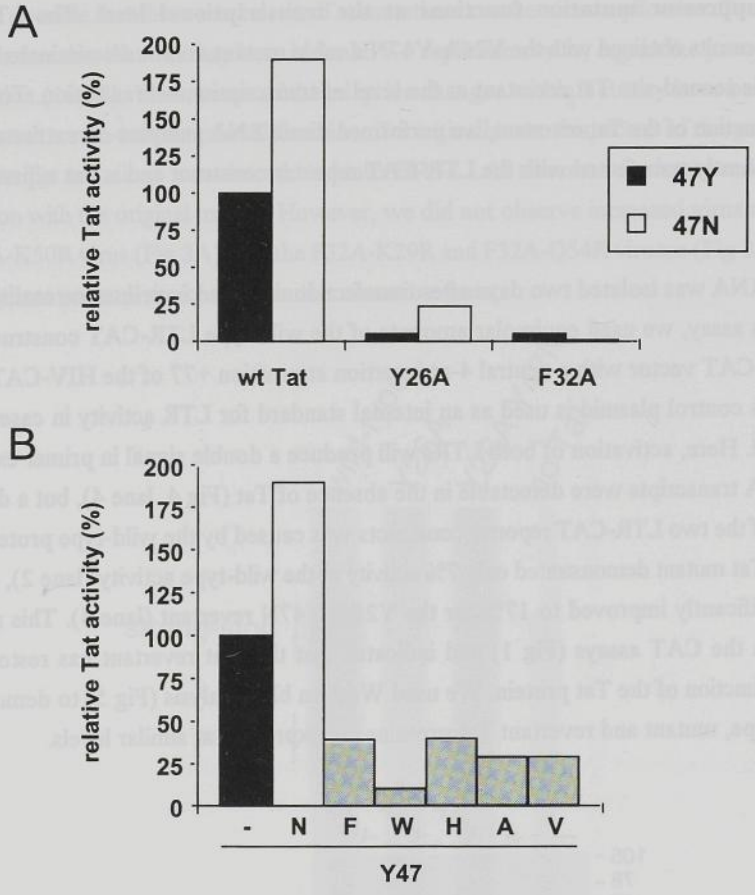


**Figure 6.** (A) Transactivation activity of several Tat proteins. Panel A compares the effect of the Y47N mutation in the context of wild-type Tat, the Y26A mutant and the F32A mutant. Panel B compares the activities of several codon 47 Tat mutants, some of the data have been published previously (41). Wild-type Tat activity is set at 100%.

**The Y47N mutation also improves the function of the wild-type Tat protein.** It is of interest to test whether the Y47N mutation does uniquely improve the function of the Y26A mutant. Such a specificity may be indicative of a direct interaction of amino acids 26 and 47 in the three-dimensional structure of Tat. We therefore introduced the Y47N mutation in the context of the wild-type Tat protein and the inactive F32A mutant. Surprisingly, LTR-CAT transfections revealed that the Y47N mutation does also improve the function of wild-type Tat to merely 200%

(Fig 6A). Thus, the Y47N mutation seems to improve the Tat function in a more general manner, independent of the Y26A mutation. Western blotting demonstrated that the Y47N Tat protein is expressed at a normal level in transfected cells (Fig 5). The positive effect of the Y47N change is even more striking if one considers the negative effects of many alternative amino acid substitutions that were tested previously at this position (summarized in Fig 6B). The Y47N mutation was not able to rescue the function of the inactive F32A mutant (Fig 6A).

Although the Y47N mutation improves the activity of the wild-type Tat protein about 2-fold, this amino acid is not observed in natural HIV-1 isolates (Table 2). We introduced the Y47N mutation in the wild-type LAI virus to test whether the viral replication rate can be improved by a more potent Tat transactivator. In replication assays, we repeatedly measured a small replication disadvantage of the Y47N mutant compared to the wild-type control (Fig 7).

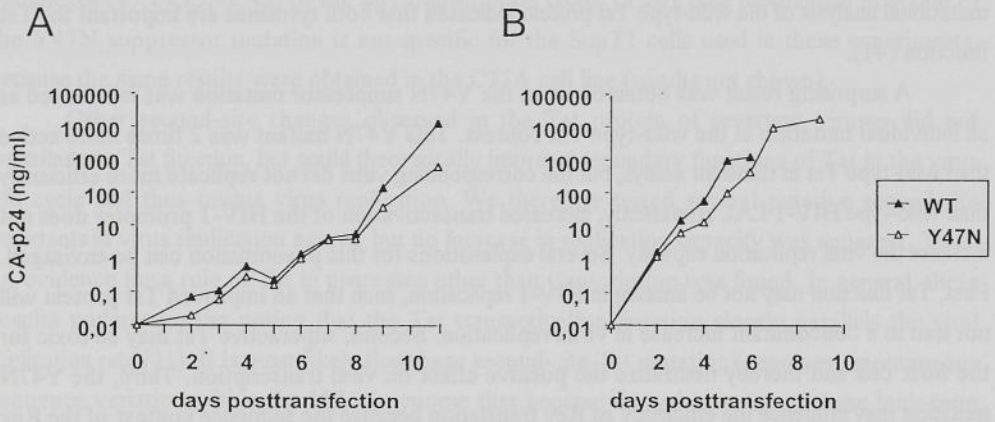


**Figure 7.** Replication kinetics of wild-type HIV-1 and the Y47N mutant virus. SupT1 cells were transfected with 0.5 μg (panel A) or 5 μg (panel B) of the molecular clones. Virus replication was monitored by measuring CA-p24 antigen production in the culture supernatant at several days posttransfection. In panel A, the culture was split at day 4 and 7 posttransfection, causing a small drop in CA-p24 values.

## Discussion

Long-term cultures were performed with different Tat-defective HIV-1 mutants to screen for second-site reversion events within the *tat* gene. Fast-replicating virus variants were observed in thirty cultures, and we subsequently analyzed the *tat* gene. We describe one Tat variant with a second-site amino acid change that restored the activity of the mutant protein. The activity of the Y26A Tat mutant was increased more than 3-fold by an additional mutation at position 47, where a tyrosine residue was replaced by asparagine (Y47N). Primer extension assays revealed that the suppressor mutation within Tat acted at the transcriptional level. It is demonstrated that the Y47N change can rescue the replication of the Y26A mutant virus. Thus, a tyrosine (Y47) was removed in response to mutation of another tyrosine (Y26). This is somewhat striking because both residues are highly conserved in natural Tat sequences, and replacement by alanine and asparagine as seen in this study is never observed in virus isolates (Table 2). Furthermore, a previous mutational analysis of the wild-type Tat protein indicated that both tyrosines are important for Tat function (41).

A surprising result was obtained when the Y47N suppressor mutation was introduced as an individual mutation in the wild-type Tat context. This Y47N mutant was 2 times more active than wild-type Tat in transient assays, but the corresponding virus did not replicate more efficiently than wild-type HIV-1 LAI. Apparently, increased transactivation of the HIV-1 promoter does not increase the viral replication capacity. Several explanations for this phenomenon can be envisaged. First, Tat function may not be limiting in HIV-1 replication, such that an improved Tat protein will not lead to a concomitant increase in virus replication. Second, superactive Tat may be toxic for the host cell and thereby neutralize the positive effect on viral transcription. Third, the Y47N mutation may influence the efficiency of Rev translation because the sequence context of the Rev initiation codon is changed (...UAUG...to...AAUG...). Because Rev production is delicately balanced to coordinate the expression of spliced and unspliced HIV-1 mRNA species, a slightly altered level of Rev protein will disturb this regulation (31). The finding that the Y47N mutation does not enhance virus fitness is consistent with the almost invariant occurrence of a tyrosine residue at this position in natural virus isolates (Table 2). Thus, the analysis of revertant viruses may demonstrate how to improve individual virus functions, but unwanted side effects are likely due to the complexity of this retroviral genome.

The loss of function that is observed for the Y26A mutant may result from aberrant folding of the mutant protein and/or from a loss of interaction of Tat with either TAR RNA or cellular cofactors. In the former scenario, the Y47N suppressor mutation could function to restore the Tat

protein structure. However, since the Y47N mutation also increased the transactivation activity of wild-type Tat, we do not think that the putative effect on the Tat structure is specific for the Y26A mutation. Preliminary structure probing experiments by limiting protease digestion of recombinant GST-Tat fusion proteins did not reveal gross differences in the degradation pattern of the wild-type Tat protein, the Y26A mutant and the Y26A-Y47N revertant (results not shown). It is not likely that TAR RNA binding is affected by the Y26A mutation, since the RNA-binding domain of Tat localizes to the basic domain (positions 49-57, (38)). However, it remains possible that the Y47N suppressor mutation, which is positioned close to the RNA-binding domain, could enhance TAR RNA binding of the Y26A mutant (and wild-type Tat). We performed Electrophoretic Mobility Shift Assays (EMSA) to study the TAR RNA interaction of the mutant and revertant Tat proteins, but no difference was observed (results not shown). Thus, we currently have no molecular explanation for the Y47N reversion event, but it is most likely that this amino acid change does influence the interaction of Tat with cellular cofactors, e.g. the PTEF-b or TFIIH complexes (15,19,23,42,45) or the Sp1 transcription factor (9,20). The mechanism of action of the Y47N suppressor mutation is not specific for the SupT1 cells used in these experiments, because the same results were obtained in the C33A cell line (results not shown).

Other second-site changes observed in the Tat protein of revertant viruses did not contribute to Tat function, but could theoretically improve secondary functions of Tat in the virus life cycle and thus rescue virus replication. We therefore tested several putative second-site revertants in virus replication assays, but no increase in replication capacity was apparent. Thus, no evidence for a role of Tat in processes other than transcription was found. In general, these results underscore the notion that the Tat transactivation function closely parallels the viral replication rate (41). It is most likely that these second-site Tat mutations represent spontaneous sequence variation within the HIV-1 genome that became fixated in some of these long-term infection experiments. Fixation of the new sequences is possible by non-random sampling effects during passage of the crippled mutants (bottle neck passage or founder effect). Alternatively, these fixated mutations may represent 'bystander mutations' that were linked to another mutation, that did improve replication and which formed the target for selection. Such a random genetic linkage is unlikely to explain the K29R mutation, which was observed 3x in independent F32A reversions and in the F38W revertant. It is possible that this particular mutation represents a frequent Tat variation. Consistent with this idea is the fact that both basic amino acids are present in natural HIV-1 isolates (7/56 R, 40/56 K, Table 2). Similarly, some of the other Tat changes that do not represent true second-site revertants are observed frequently in natural isolates (e.g. Q54R 5/56).

We do not understand how the Y47H virus has gained replication capacity since this particular mutant is also Rev-defective. The fixated second-site Tat mutation Q17K seems unable to restore Rev expression, but analysis of the more important second coding exon of Rev in the revertant virus may provide more information. Alternatively, this revertant virus may actually represent a Rev-independent HIV-1 variant. These possibilities are currently under investigation.

A surprising result of this evolution study with Tat-mutated viruses is that virus replication can apparently be restored by changes that map outside the Tat first coding exon. This is the case for revertant viruses that do not contain compensatory mutations within Tat, but also for Tat proteins with second-site mutations that do not improve virus fitness. We cannot exclude that suppressor mutations are present in the second Tat coding exon that encodes the C-terminal 14 amino acids. However, we do not expect such mutations in this part of the protein, since the C-terminus of Tat contributes only marginally to virus replication (28,39). *A priori*, it would seem possible to repair a defect in the Tat transcriptional activator by second-site changes in the LTR promoter. In particular, one would expect LTR changes that improve the basal promoter activity, thus rendering the virus less dependent on Tat. We have initiated studies to screen for such LTR adaptations. It is also possible that improvement of other, unrelated viral functions can partially restore the replication of Tat-mutated viruses. For instance, we reported recently that a translation-impaired HIV-1 mutant can dramatically improve its replication by optimizing the mechanistically unrelated Env function (12). This example underscores the notion that the outcome of virus evolution studies can sometimes be very complex, yet intriguing.

One could argue that the forced evolution approach used here for the recovery of spontaneous revertant viruses is not very effective, since only 1 true second-site Tat revertant was obtained out of a total of 30 cultures that harbored a fast-replicating virus revertant. However, we did isolate 11 first-site Tat revertants, and some were generated by relatively difficult types of mutation (e.g. multiple transversions). These results suggest that there may be very few single amino acid changes at secondary sites that can render the Tat mutants active. This finding suggests the importance of specific residues at these positions, and these amino acids may be involved in interactions with cellular cofactors or the TAR RNA element. Alternatively, these amino acids may be required to form the specific tertiary structure of the Tat activation domain. In this scenario, the conformation apparently cannot be restored by any amino acid change elsewhere in the protein. In addition, the presence of overlapping reading frames (*vpr*, *rev*) and RNA signals (splice acceptors, splicing silencers (1)) may put additional constraints on the evolutionary flexibility of the *tat* gene (28). Thus, the evolutionary approach may not be an efficient method to study structure-function relationships in small proteins with a high information density. Obviously, we

cannot exclude the possibility that the activity of these Tat mutants can be restored by multiple amino acid changes at secondary sites, but the probability of finding such hypermutated variants is remote. Perhaps the virus evolution experiment can benefit from strategies to introduce random mutations within the gene under selection (24,35). Alternatively, part of the *tat* gene can be provided as a randomized nucleotide sequence (5), such that the search for second-site revertants will occur in a much broader section of sequence space. A major disadvantage of these approaches is that a significant fraction of viral genomes that are manipulated in this way will contain additional mutations that interfere with virus replication.

The forced evolution approach should be applicable to other virus genes, and perhaps larger proteins/enzymes are more amenable for second-site repair (37,43). We and others demonstrated previously that this approach is ideally suited to study RNA elements, in particular structured motifs that play critical roles in virus replication (6,21,29,30). The enormous genetic flexibility of structured RNA motifs is due to the fact that completely different sequences can form very similar basepaired structures. The relative inflexibility of the Tat protein may make it an excellent target for the development of potent anti-HIV drugs.

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# Chapter 7

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# Evolution of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Promoter by Conversion of an NF-κB Enhancer Element into a GABP Binding Site

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**Human immunodeficiency virus type 1 (HIV-1) transcription is regulated by the viral Tat protein and cellular factors, of which the concentration and activity may depend on the cell type. Viral long terminal repeat (LTR) promoter sequences are therefore optimized to suit the specific nuclear environment of the target host cell. In long-term cultures of a Tat-defective, poorly replicating HIV-1 mutant, we selected for a faster-replicating virus with a 1-nucleotide deletion in the upstream copy of two highly conserved NF-κB binding sites. The variant enhancer sequence demonstrated a severe loss of NF-κB binding in protein binding assays. Interestingly, we observed a new binding activity that is specific for the variant NF-κB sequence and is present in the nuclear extract of unstimulated cells that lack NF-κB. These results suggest that inactivation of the NF-κB site coincides with binding of another transcription factor. Fine mapping of the sequence requirements for binding of this factor revealed a core sequence similar to that of Ets binding sites, and supershift assays with antibodies demonstrated the involvement of the GABP transcription factor. Transient transfection experiments with LTR-chloramphenicol acetyltransferase constructs indicated that the variant LTR promoter is specifically inhibited by GABP in the absence of Tat, but this promoter was dramatically more responsive to Tat than the wild-type LTR. Introduction of this GABP site into the LAI virus yielded a specific gain of fitness in SupT1 cells, which contain little NF-κB protein. These results suggest that GABP potentiates Tat-mediated activation of LTR transcription and viral replication in some cell types. Conversion of an NF-κB into a GABP binding site is likely to have occurred also during the worldwide spread of HIV-1, as we noticed the same LTR modification in subtype E isolates from Thailand. This typical LTR promoter configuration may provide these viruses with unique biological properties.**

Human immunodeficiency virus type 1 (HIV-1) transcription is directed by the promoter located in the 5′ long terminal repeat (LTR) of the integrated provirus. Transcription is controlled both by cellular factors that bind to enhancer elements in the U3 region of the LTR and by the virally encoded Tat protein (reviewed in reference 29). The Tat protein transactivates the HIV-1 promoter several hundred-fold and is essential for virus replication. Tat binds an RNA hairpin, termed TAR, that is present at the 5′ end of all viral transcripts (8, 15). This unique Tat protein-TAR RNA complex stimulates transcription by recruiting a cyclin–cyclin-dependent kinase complex to the promoter that phosphorylates the C-terminal domain of RNA polymerase II (25, 27, 29, 60).

The U3 enhancer region of the LTR promoter contains binding sites for the Sp1 and NF-κB transcription factors. Both Sp1 and NF-κB are constitutively expressed, but the latter factor is present as an inactive complex with IκB protein in the cytoplasm of unstimulated cells. Dissociation of this complex and migration of active NF-κB into the nucleus can be induced by a large number of extracellular stimuli (52, 56). Such stimuli include infection by some viruses, phorbol esters, and multiple cytokines. NF-κB is a heterodimer composed of two proteins of the Rel/κB family of transcription factors, p50 and RelA (52). NF-κB recognizes a 10-bp stretch of DNA with the consensus sequence 5′-GGGPuNNPyPyCC-3′ (52). Both the p50 and RelA subunits are required for DNA binding, but the transactivation domain is provided by RelA (33).

The two tandem NF-κB binding sites in the HIV-1 LTR are highly conserved among different viral isolates, suggesting an essential role in virus replication. Consistent with this idea, mutation of either NF-κB site resulted in a dramatic loss of LTR promoter activity in transient transfection studies with LTR-CAT reporter constructs (7, 42). Mutations of the NF-κB binding sites in infectious HIV-1 clones yielded conflicting results regarding their contribution to virus replication. Initial studies indicated that the NF-κB enhancer elements are dispensable for virus growth (36, 45), but more recent analyses demonstrated the importance of these elements for optimal HIV-1 replication (1, 11). Interestingly, a direct correlation was observed between the severity of the replication defect of NF-κB site-mutated viruses and the NF-κB protein level of the cell type used for infection (11, 36).

As part of an analysis of Tat protein structure and function, we constructed a set of HIV-1 molecular clones with a mutant Tat protein. Several replication-impaired HIV-1 mutants with a defective Tat function were described previously (58). To select for revertant viruses with improved replication capacity, we maintained the transfected cell cultures for a prolonged period. The *tat* gene of several, but not all, revertant viruses demonstrated first- or second-site amino acid changes that explain the reversion event (unpublished data). The LTR pro-

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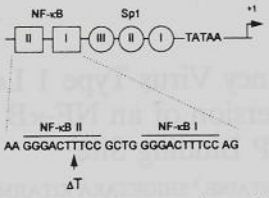


FIG. 1. Scheme of the HIV-1 LTR promoter-enhancer. The NF-κB and Sp1 binding sites are indicated by boxes and circles, respectively. The TATAA motif and the start site of transcription (+1) are indicated. The nucleotide sequences of the two NF-κB enhancers are shown. In a revertant of a Tat-mutated virus, a single T deletion (ΔT) was observed in the upstream NF-κB site II.

moter region was also analyzed for mutations that may improve transcription of a Tat-defective virus through modulation of the promoter-enhancer elements. In this study, we describe one revertant virus with a single-nucleotide deletion in the upstream NF-κB enhancer (Fig. 1) that is predicted to abolish NF-κB binding (33, 52). Because a virus with a loss of enhancer function is unlikely to repair a Tat defect, it was anticipated that a binding site for another transcription factor could have been generated. Indeed, such a gain of function was apparent in protein binding studies with the variant NF-κB site. It is demonstrated that the GABP protein of the Ets family of transcription factors binds to the variant LTR sequence. This tissue culture evolution experiment not only underscores the enormous genetic flexibility of HIV-1 but also indicates that virus variants with modified LTR promoter-enhancer motifs that may respond differently to cellular activation signals can evolve. Interestingly, we observed the same enhancer switch in HIV-1 isolates that belong to subtype E.

## MATERIALS AND METHODS

**Cloning and sequencing of proviral 3′ LTR segments.** Chromosomal DNA from HIV-infected cells was isolated as described elsewhere (12). Proviral 3′ LTR sequences were amplified in a standard 35-cycle PCR using the 5′ primer Nefseq1 (5′ ATTCGCCACATACCTAGAAG 3′, proviral positions 8789 to 8808) and the 3′ U5 primer C(N1) (5′ GGTCTGAGGGATCTCTAGTTACC AGAGTC 3′, proviral positions 9737–9709). The 948-bp PCR product was gel purified and digested with *Xho*I (position 8944) and *Hin*dIII (position 9663), and the resulting 719-bp fragment was cloned into pBlue 3′ LTR-CAT (30) to replace the wild-type LTR sequences. These constructs were used as LTR-chloramphenical acetyltransferase (CAT) reporter plasmids in transient transfection assays. Clones were sequenced by using ET Dyeprimer technology (Amersham) on an Applied Biosystems 373 DNA sequencer.

**Transient transfections and CAT assays.** The T-cell lines SupT1 and Jurkat were cultured in RPMI medium supplemented with 10% fetal calf serum and penicillin (100 U/ml)-streptomycin (100 μg/ml). Transient transfection of these cells was carried out by means of electroporation as described previously (31). The cell lines were transfected with the indicated amounts of LTR-CAT reporter plasmid to determine basal promoter activity. The activated promoter activity was measured in transfections with 1 μg of LTR-CAT and 2.5 μg of pcDNA3-Tat (58), unless indicated otherwise. GABPα and -β1 expression plasmids (44) were used at 1.8 μg per transfection. In some experiments, cells were treated with phorbol myristate acetate (PMA; 25 ng/ml)-phytohemagglutinin (PHA; 1 μg/ml) or tumor necrosis factor alpha (TNF-α; 30 ng/ml) at 24 h posttransfection. Cell lysates for CAT assays were prepared at 3 days posttransfection as described (58) and CAT assays were performed in the linear range of the assay by the phase extraction method (49). The Tat response was calculated as the ratio of activated over basal LTR activity, with a correction for the different amounts of LTR-CAT plasmid used in the transfections with and without Tat. This correction is valid, because the basal transcription level depends on the quantity of LTR-CAT plasmid in a linear manner up to 40 μg in this transfection system (results not shown).

**EMSA.** Nuclear extracts from HeLa and SupT1 cells were prepared essentially as described previously (14). For some extracts, the cells were treated prior to extraction with TNF-α (30 ng/ml) for 15 min at 37°C to induce NF-κB binding activity in the nucleus. The oligonucleotides used in the electrophoretic mobility shift assays (EMSAs) are listed in Table 1. We hybridized 250 pmol of sense oligonucleotide to 250 pmol of the corresponding antisense oligonucleotide in 100 μl of buffer consisting of 10 mM Tris (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, and 10% glycerol. The samples were heated at 90°C for 4 min and slowly cooled to room temperature. End labeling was performed for 30 min at 37°C with 2.5 pmol of double-stranded DNA, [γ-$^{32}$P]ATP, and T4 polynucleotide kinase according to the protocol of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). The reaction was stopped with 1 μl of 0.5 M EDTA, and free label was removed on a Sephadex G50 column. EMSAs were performed with 5 μg of HeLa or 2 μg of SupT1 nuclear extract–0.5 μg of poly(dI-dC)–10 mg of bovine serum albumin in a 20-μl reaction mixture containing 10 mM Tris (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM dithiothreitol. The labeled probe (30,000 to 50,000 cpm) was added to this mixture, and the sample was incubated for 25 min at room temperature. In competition assays, we added 1 pmol of unlabeled double-stranded oligonucleotide to the reaction before addition of the labeled probe (approximately 20 fmol). In supershift EMSAs, we preincubated the EMSA sample for 5 min, after which 1 μl of antiserum was added and incubation was continued for an additional 20 min at room temper-

TABLE 1. EMSA oligonucleotides

| Name | Sequence (5′ to 3′) | |
|---|---|---|
| | Sense | Antisense |
| wt | AAGGGACTTTCCGC | GCGGAAAGTCCCTT |
| mut | AAGGGACTTCCGC | GCGGAAGTCCCTT |
| wt-wt | AAGGGACTTTCCGCTGGGGACTTTCCAG | CTGGAAAGTCCCCAGCGGAAAGTCCCTT |
| mut-wt | AAGGGACTTCCGCTGGGGACTTTCCAG | CTGGAAAGTCCCCAGCGGAAGTCCCTT |
| d3-11 | AAGGGACTTCCGCTGG | CCAGCGGAAGTCCCTT |
| d3-8 | AAGGGACTTCCGCTGGGGA | TCCCCAGCGGAAGTCCCTT |
| d3-5 | AAGGGACTTCCGCTGGGGACTT | AAGTCCCCAGCGGAAGTCCCTT |
| d3-2 | AAGGGACTTCCGCTGGGGACTTTCC | GGAAAGTCCCCAGCGGAAGTCCCTT |
| d5-3 | GGACTTCCGCTGGGGACTTTCCAG | CTGGAAAGTCCCCAGCGGAAGTCC |
| d5-6 | CTTCCGCTGGGGACTTTCCAG | CTGGAAAGTCCCCAGCGGAAG |
| d5-9 | CCGCTGGGGACTTTCCAG | CTGGAAAGTCCCCAGCGG |
| MD-wt | GGACTTTCCGCTGGGGA | TCCCCAGCGGAAAGTCC |
| MD-mut | GGACTTCCGCTGGGGA | TCCCCAGCGGAAGTCC |
| MD-A | AAACTTCCGCTGGGGA | TCCCCAGCGGAAGTTT |
| MD-B | GGGTTTCCGCTGGGGA | TCCCCAGCGGAAACCC |
| MD-C | GGACCCCCGCTGGGGA | TCCCCAGCGGGGGTCC |
| MD-D | GGACTTTTGCTGGGGA | TCCCCAGCAAAAGTCC |
| MD-E | GGACTTCCATTGGGGA | TCCCCAATGGAAGTCC |
| MD-F | GGACTTCCGCCAGGGA | TCCCTGGCGGAAGTCC |
| MD-G | GGACTTCCGCTGAAGA | TCTTCAGCGGAAGTCC |
| MD-H | GGACTTCCGCTGGGAG | CTCCCAGCGGAAGTCC |

ature. The complexes were resolved on nondenaturing 4% polyacrylamide gels in 0.25× Tris-borate-EDTA at 200 V for 2 h. Gels were dried and exposed to X-ray film at −70°C. Quantitation of EMSA signals was performed with a Molecular Dynamics PhosphorImager and ImageQuant software.

**Protein and antibody reagents.** Purified recombinant NF-κB p50 and p52 (p49) were obtained from Promega, Madison, Wis. Monoclonal antibodies against the RelA and p50 NF-κB subunits were purchased from Rockland, Gilbertsville, Pa. The preimmune sera and antisera against the GABP α and β1 subunits were described previously (28).

**Construction of the variant LAI virus and replication studies.** The GABP promoter region was cut from the pBlue 3′ LTR-CAT vector with *Bsp*EI and *Bfr*I and cloned into plasmid pBlue 3′ LTR (30), thus replacing the wild-type sequence. This plasmid was used to replace the wild-type 3′ LTR in pLAI by exchange of the *Xho*I-*Bgl*I fragment as described previously (30). All constructs were verified by sequence analysis.

C33A cells were cultured and transfected as described previously (13). Viral stocks were prepared by transfection of C33A cells with 1 μg of the wild-type plasmid pLAI and the GABP variant in 24-well multidish plates. The supernatant was taken at 2 days posttransfection and frozen at −70°C in aliquots. CA-p24 levels were determined by antigen capture enzyme-linked immunosorbent assay as described previously (2), and these values were used to normalize the amount of virus in subsequent infection assays on susceptible cells.

MT-2 and C8166 cells were cultured as described above for the SupT1 cell line. Infections were performed with $10^6$ cells in 1 ml of RPMI medium with Polybrene (5 μg/ml) for 1 h at 37°C. The culture volume was subsequently increased to 5 ml with RPMI medium. Peripheral blood mononuclear cells (PBMC) were cultured as described elsewhere (2), and $5 \times 10^6$ cells were used for infection as described above. Cells were infected with an amount of virus indicated in the figure legends. All cultures were monitored for CA-p24 production following infection.

Mixed infections of SupT1 cells with an equal amount of the wild-type and GABP virus (8 ng of CA-p24 each) were performed to determine the relative fitness of the two viruses. At the peak of infection, as indicated by the presence of massive syncytia, virus was passaged onto fresh SupT1 cells and virus replication was continued for 40 days. Samples of infected cells were taken at different days, and chromosomal DNA was isolated as described previously (12). Proviral LTR sequences were PCR amplified as described above, using primers XhoU3 (5′ CCGCTCGAGTGGAAGGGCTAATTCACT 3′, proviral positions 9133 to 9150) and C(N1). Population-based sequencing was performed with the 3′ TATA primer (5′ GCAAAAAGCAGCTGCTTATATGCA 3′, proviral positions 9578 to 9555) and DyeTerminator sequencing technology (Amersham) on an ABI automated sequencer. The sequence of both HIV-1 isolates is identical up to the ΔT mutation in the NF-κB site but will be read in different reading frames downstream of this position. Overlapping sequence signals representing the two viruses were quantitated at several positions to determine the ratio of wild-type to variant virus.

## RESULTS

**Selection of an HIV-1 variant with an unusual LTR mutation.** We previously described several replication-impaired HIV-1 mutants with a single amino acid substitution in the cysteine-rich domain of the Tat protein (58). Two Tat-mutated viruses (Tyr26Ala and Phe32Ala) were chosen for the selection of revertant viruses in several SupT1 T-cell cultures that were maintained for over 4 months. The rationale behind this forced evolution approach is to identify second-site mutations within the Tat protein that are able to restore the function of this small transactivator protein. Although rapidly replicating viruses appeared in most of the cultures, only some of these revertant viruses contained either first-site or second-site Tat mutations (unpublished data). To identify putative adaptive changes in the HIV-1 promoter-enhancer motifs and the TAR element that constitutes the Tat binding site, we analyzed the sequence of the LTR region of each of these revertant viruses. One culture that was infected with the Tyr26Ala mutant yielded a fast-replicating virus that maintained the original Tat mutation but acquired a remarkable sequence variation in the upstream NF-κB motif that is predicted to abolish NF-κB binding (Fig. 1, site II). Because it is difficult to understand how the loss of NF-κB binding can assist a Tat-defective virus, we reasoned that perhaps a binding site for another transcription factor was generated by this mutation.

**The LTR sequence variation results in a loss of NF-κB binding.** First, we analyzed the effect of the 1-nucleotide (nt)

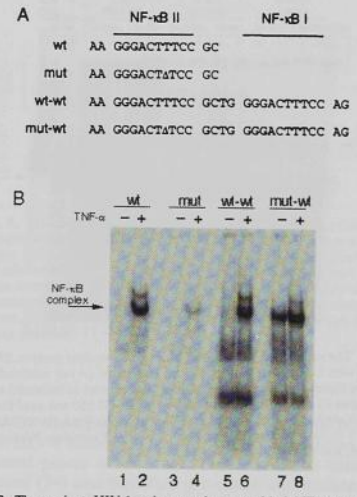


FIG. 2. The variant HIV-1 enhancer does not bind NF-κB protein. (A) Sequences of the probes used in the EMSA (plus strand only is shown). Probes wt and mut represent the wild-type and variant (ΔT) NF-κB site II, flanked by two nucleotides on each side. Probes wt-wt and mut-wt also contain the downstream NF-κB site I. (B) EMSA with nuclear extract from HeLa cells treated with (+) or without (−) TNF-α to induce NF-κB binding activity. The probes used in EMSA are indicated above the lanes. The signal corresponding to the NF-κB complex is indicated by an arrow.

deletion in the NF-κB site II on protein binding in EMSA with double-stranded DNA oligonucleotides and nuclear extract from HeLa cells. Since NF-κB is an inducible transcription factor, we also prepared extracts from HeLa cells that were stimulated with TNF-α. The oligonucleotides used for EMSA represent the NF-κB site II, either the wild-type or mutant sequence, with two flanking base pairs on each side (Fig. 2A, wt and mut). As shown in Fig. 2B, stimulation of HeLa cells with TNF-α resulted in the induction of NF-κB binding activity in the nucleus (lanes 1 and 2), and this binding was largely abolished for the mutant NF-κB site (lane 4). This result indicated a loss of NF-κB binding function for the mutant HIV-1 promoter. Quantitation of the signals in lanes 2 and 4 demonstrated an eightfold decrease in NF-κB binding for the mutant LTR motif. When larger oligonucleotides including the downstream NF-κB site I were used (Fig. 2A, wt-wt and mut-wt), a somewhat different binding pattern was observed. The binding properties of the wt-wt probe (lanes 5 and 6) is identical to that of the shorter wt probe (lanes 1 and 2). However, a new binding activity was detected with the mut-wt probe in unstimulated HeLa nuclear extract (lane 7). This activity is specific for the mutant sequence, as it is not observed with the wt-wt probe (lane 5). Interestingly, this new protein-DNA complex was not observed in EMSA with the shorter mut probe (lane 3), indicating that sequences downstream of NF-κB site II are required for binding of this new factor that is present in nuclear extract of unstimulated cells. The mut-wt probe demonstrated increased protein binding in stimulated nuclear extract (lane 8). This most likely represents NF-κB binding to the intact site I. However, because the new protein-DNA complex comigrated with the regular NF-κB complex, it is not possible to distinguish between these two complexes in

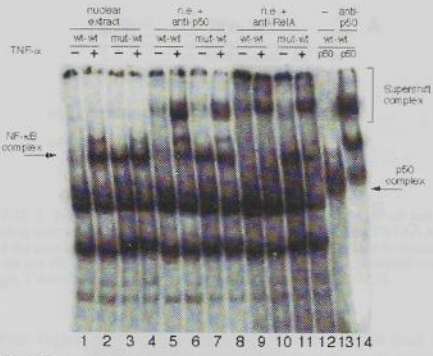


FIG. 3. The variant HIV-1 enhancer binds a new nuclear protein. EMSA was performed with HeLa nuclear extract (n.e.) stimulated (+) or unstimulated (–) with TNF-α (lanes 1 to 12) and probes wt-wt and mut-wt as indicated above the lanes. In lanes 13 and 14, purified recombinant NF-κB p50 was used for EMSA. Monoclonal antibodies specific for the NF-κB subunits RelA (lanes 9 to 12) and p50 (lanes 5 to 8) were used to supershift the NF-κB complex. Positions of the NF-κB, p50, and supershift complexes are indicated.

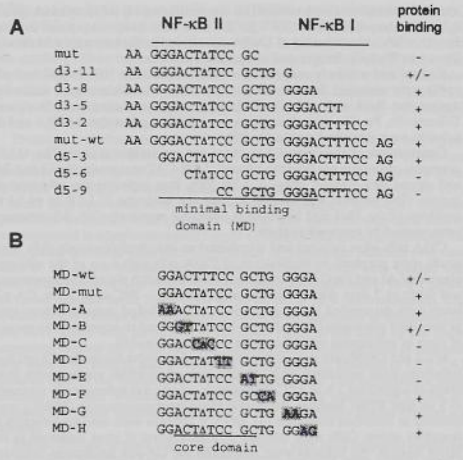


FIG. 4. Mutational analysis of the new protein binding site. (A) A set of 5′- and 3′-deleted probes was tested in EMSA for the ability to compete with probe mut-wt for protein binding in unstimulated nuclear extracts. Efficient competition (and thus protein binding) is scored as +; no binding is scored as –. The MD, which overlaps both NF-κB sites, is underlined. (B) Determination of the sequence requirements for GABP binding. Mutant probes (MD-A to -H; substituted nucleotides are shaded) were tested for the ability to compete for protein binding competition to the MD-mut probe in EMSA. The sequence-specific core of 8 nt is underlined.

this experiment. The faster-migrating bands observed with the extended wt-wt and mut-wt probes (lanes 5 to 8) represent aspecific protein-DNA complexes that are frequently observed in NF-κB EMSA (48, 61).

The NF-κB protein is a p50-RelA (p65) heterodimeric complex, but numerous alternative homo- or heterodimers can be formed by different members of the Rel family of transcription factors. Each complex displays a different DNA binding specificity and transactivation potential (52, 56), and it is therefore feasible that the mutant NF-κB site has been optimized to accommodate the binding of an alternative NF-κB complex that is distinct from p50-RelA. For instance, the p52-RelA heterodimer has been suggested to be involved in specific transactivation of the HIV-1 promoter (47, 48). To investigate this possibility, we performed EMSA in the presence of antibodies directed against the NF-κB subunits (Fig. 3). The new binding activity, which is present in unstimulated nuclear extract and specific for probe mut-wt, is shown in lane 3. Lanes 1, 2, and 4 represent the appropriate control experiments; the regular NF-κB complex is, for instance, detected with probe wt-wt and stimulated nuclear extract (lane 2). The same four EMSA samples were incubated with an anti-p50 antibody (lanes 5 to 8) and a RelA-specific antibody (lanes 9 to 12). Incubation with anti-p50 supershifted the NF-κB complex (lane 6), but the new protein-DNA complex is not recognized by this antiserum (lane 7). As a control for the specificity of this antiserum, it is demonstrated that a complex made with probe wt-wt and purified recombinant p50 protein can be supershifted with this monoclonal antibody (lanes 13 and 14). Similar results were obtained with the RelA-specific antibody: an NF-κB supershift (lane 10) but no effect on the new DNA-protein complex (lane 11). Interestingly, the protein complex observed with probe mut-wt in stimulated nuclear extract is only partially supershifted by both NF-κB antisera (lanes 8 and 12). Since the amount of antibody used is sufficient to efficiently supershift the NF-κB signal obtained with probe wt-wt (lanes 6 and 10), we propose that the unreactive signal with probe mut-wt represents the new protein-DNA complex. In fact, this result may suggest that the new factor and NF-κB are not simultaneously bound to probe mut-wt.

We also analyzed the binding of the wt-wt and mut-wt DNA probes to recombinant NF-κB homodimer forms. The p50-p50 homodimer bound both probes (not shown), in line with the observation that binding of p50 requires the 5′ half of the NF-κB recognition sequence (33), which is not affected by the T deletion. The p52-p52 homodimer did not bind to any of the probes, consistent with the low binding affinity of p52 for the HIV-1 NF-κB sites (47). These combined results indicate that the factor that binds the variant NF-κB II site is not related to the Rel family of transcription factors.

**Fine mapping of the binding site for the new protein factor.** To further analyze the factor that binds specifically to the modified LTR sequence, we first determined the minimal DNA sequence requirements. Because binding was observed with probe mut-wt but not with probe mut (Fig. 2B), it is possible that sequences downstream of the NF-κB site II contribute to protein binding. To test this, a nested set of 5′/3′-truncated probes was synthesized and analyzed for the ability to compete with probe mut-wt for binding of the new protein factor in EMSA with unstimulated cell extracts (Fig. 4A). Deletion of 2, 5, and 8 nt from the 3′ end of probe mut-wt did not influence protein binding (d3-2, d3-5, and d3-8). Deletion of 11 nt partially affected binding (d3-11), and deletion of 14 nt abrogated binding (d3-14, the original mut probe). Removal of 3 nt from the 5′ end of probe mut-wt showed no effect (d5-3), but deletion of 6 and 9 nt abolished protein binding (d5-6 and d5-9). These results suggest that the minimal DNA binding domain (MD) constitutes the sequence GGACTTCCGCTGG GGA, which overlaps both NF-κB sites (Fig. 4A).

We next synthesized this MD and demonstrated protein binding for the mutant LTR sequence. Scanning mutagenesis was used to replace all nucleotides of this MD-mut probe by

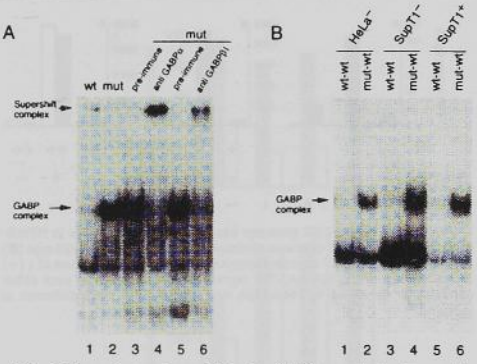


FIG. 5. The variant enhancer binds the GABP transcription factor. (A) EMSA was performed with probes wt-wt (lane 1) and mut-wt (lanes 2 to 6). The latter sample was supershifted with rabbit polyclonal antiserum specific for the α and β1 subunits of GABP or the control preimmune sera. Positions of the GABP and supershift complexes are indicated. (B) EMSA was performed with probes wt-wt and mut-wt in nuclear extracts of unstimulated HeLa cells (HeLa$^-$) and unstimulated and stimulated SupT1 cells (SupT1$^-$ and SupT1$^+$). The position of the GABP complex is indicated.

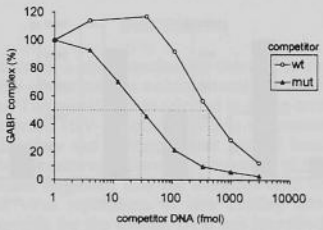


FIG. 6. Mutation of the NF-κB enhancer greatly stimulates GABP binding. The relative affinity of GABP for the MD-mut and MD-wt probes was measured as follows. EMSA was performed with unstimulated HeLa nuclear extract and the $^{32}$P-labeled MD-mut probe, and unlabeled MD-mut and MD-wt probes were added as competitor in the range of 4 to 3,000 fmol. The GABP complex was quantitated with a PhosphorImager and plotted (signal in the absence of competitor set at 100%). The dotted lines mark the position at which 50% competition was observed.

transitional mutation in a pairwise manner (Fig. 4B, MD-A to -H). This set of MD probes was tested for the ability to compete with the MD-mut probe for protein binding. Mutation of two 5′-terminal G residues in probe MD-A did not affect protein binding. Binding was partially lost upon mutation of the AC dinucleotide in probe MD-B and was abrogated for mutants MD-C, MD-D, and MD-E. The 3′-terminal sequence of the MD-mut probe does not contribute to protein binding in a sequence-specific manner, as efficient competition was measured for the mutants MD-F, MD-G, and MD-H. These results are summarized in Fig. 4B, where the core domain that provides important sequence information is underlined.

**The variant LTR gains GABP binding activity.** The core domain ACTTCCGC is reminiscent of the binding site for Ets transcription factors because the noncoding strand contains the GGA motif that is essential for Ets factor binding (24). Unlike most Ets binding sites, the mutant HIV-1 LTR has the GGA motif in the noncoding strand. The thrombopoietin gene is one example of a cellular gene with the HIV-like orientation of an Ets binding site in its promoter (28). This Ets site binds GABP, and the core of this GABP binding site (ACTTCCG) is identical to that of the variant HIV-1 sequence. These combined observations raised the possibility that GABP is the factor that binds the modified HIV-1 LTR. GABP is a heterodimer consisting of an α and a β subunit of 60 and 53 kDa, respectively. GABPα is an Ets family transcription factor with a DNA binding Ets domain; GABPβ1 is a heterotypic protein related to *Drosophila melanogaster* Notch and contains a series of ankyrin repeats that interact with GABPα (34). Formation of the heterodimer enhances DNA binding and specificity (5).

To test whether the T deletion in the NF-κB site II facilitates GABP binding, we performed EMSA with rabbit polyclonal antibodies directed against the α and β subunits of GABP. Figure 5A shows the protein-DNA complex that is specific for probe mut-wt (lane 2). Incubation of this EMSA sample with preimmune sera demonstrated no effect (lanes 3 and 5), but the DNA-protein complex was supershifted with both anti-GABP antibodies (lanes 4 and 6). This experiment identified the GABP transcription factor as the protein that binds to the variant HIV-1 enhancer element. This idea is supported by two additional pieces of evidence. First, GABP is constitutively expressed (34) and therefore present in unstimulated nuclear extract. Second, the GABP heterodimer is similar in size to the NF-κB dimer, which is consistent with the similar migration of the corresponding DNA complexes on EMSA gels.

GABP is expressed in many cell types, including the HeLa cells that were used for preparation of the nuclear extract (34). Since the HIV-1 revertant with the variant LTR was selected upon long-term replication in the SupT1 T-cell line, we expected GABP to be present in the nuclear extract of these cells as well. To test this, we performed EMSA with nuclear extract from unstimulated and TNF-α-stimulated SupT1 cells (Fig. 5B, SupT1$^-$ and SupT1$^+$, respectively). For comparison, the unstimulated HeLa nuclear extract (HeLa$^-$) was included to demonstrate specific GABP binding to probe mut-wt (Fig. 5B, lane 2). Indeed, a complex with the gel mobility of the DNA-GABP complex was detected both in unstimulated and stimulated SupT1 nuclear extracts (lanes 4 and 6). This binding was specific for the variant LTR probe. Note the absence of NF-κB binding activity in the stimulated SupT1 nuclear extract, which should have reacted with probe wt-wt (lane 5). This is consistent with the observation that stimulated SupT1 cells contain extremely low levels of NF-κB (11).

The results so far indicate a gain of GABP binding upon mutation of the HIV-1 LTR sequence, but there is some evidence that the GABP transcription factor can also bind with a low affinity to the wild-type HIV-1 promoter. First, such an effect was described by Flory and coworkers (17). Second, we measured some GABP binding with the MD-wt probe, which has the wild-type NF-κB sequence (Fig. 4B). To analyze the relative binding affinity of GABP for the MD-wt and MD-mut probes, we performed a competition experiment. EMSA was performed with the labeled MD-mut probe and unstimulated HeLa nuclear extract. Either the MD-mut or MD-wt probe was added in increasing amounts as competitor. The GABP bandshift signals were quantitated on a PhosphorImager, and the results are presented in Fig. 6. The EMSA signal in the absence of competitor was set at 100%, and the relative EMSA signals were plotted as a function of the amount of competitor DNA. It is obvious that the MD-mut probe is a much more efficient competitor than the MD-wt probe. A 14-fold increase in relative binding affinity was calculated by comparing the

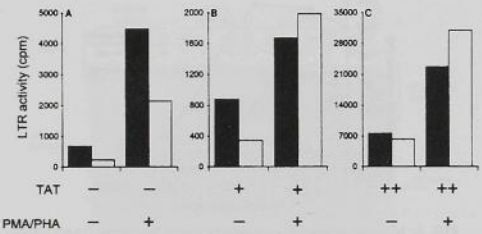


FIG. 7. The variant LTR promoter combines low basal activity with high Tat inducibility. SupT1 T cells were electroporated with 40 (A) or 1 (B and C) μg of wild-type (■) or mutant (□) LTR-CAT reporter plasmid. Some samples were cotransfected with 1 (+) or 3 (++) μg of the Tat expression plasmid and treated with PMA-PHA 24 h posttransfection as indicated. Transfections were performed simultaneously, and results of a representative experiment are shown. Similar results were obtained in five independent transfection experiments.

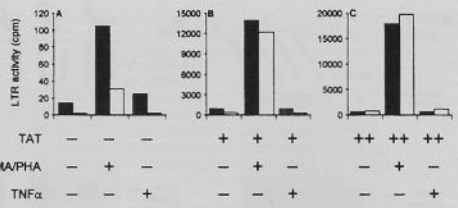


FIG. 8. The variant LTR promoter has the same activity profile in NF-κB-containing cells. Jurkat T cells were electroporated with 1 μg of the wild-type (■) or mutant (□) LTR-CAT reporter construct in the absence or presence of 1 (+) or 2 (++) μg of the pcDNA3-Tat expression vector. The cells were either untreated or stimulated by PMA-PHA or TNF-α at 24 h posttransfection as indicated.

amounts of competitor that reduce the EMSA signal by 50%: 30 fmol for MD-mut and more than 400 fmol for MD-wt.

**The GABP binding site changes the activity of the HIV-1 LTR promoter.** To perform transcription studies, we inserted the wild-type and variant LTR promoters upstream of the CAT reporter gene. To accurately measure the extremely low basal promoter activity, 40 μg of LTR-CAT plasmid was transfected into SupT1 cells. Gene expression with the variant LTR promoter was less than half of that with the wild-type LTR promoter (Fig. 7A). The mitogens PMA and PHA stimulate signal transduction pathways and can activate NF-κB expression in the nucleus (52). In addition, PMA activates the tyrosine kinase/Ras/Raf signaling pathway, leading to enhanced expression from promoters that are regulated by Ets transcription factors (6). Specifically, it has been demonstrated that GABP subunits are phosphorylated by the mitogen-activated protein kinase/ERK kinase pathway upon treatment of cells by phorbol ester, and such a posttranslational modification may regulate the transcriptional activity of GABP (17). We therefore determined the effects of these stimuli on the wild-type and mutant LTRs. The addition of PMA and PHA greatly increased basal activities of both the wild-type and mutant promoters, but the latter remained twofold less active than the former.

Transactivation of the LTR promoter by the viral Tat protein was assayed in transfections with 1 μg of LTR-CAT and 1 or 3 μg of Tat plasmid (Fig. 7B and C). Both situations were tested in the absence and presence of PMA-PHA. Both promoters demonstrate an approximately 50-fold Tat induction compared with an LTR-CAT transfection in the absence of Tat (not shown). The variant LTR remained less active than the wild-type LTR at low Tat levels, but this difference was less prominent at high Tat levels, and the mutant promoter activity exceeded that of the wild-type LTR in the presence of Tat and PMA-PHA. These results show that replacement of the upstream NF-κB site by a GABP element can have a positive effect on the HIV-1 gene expression level under certain conditions. This result is striking compared with the behavior of other NF-κB knockout mutations, which show a severe reduction in LTR promoter activity (reference 7 and data not shown).

The loss of the NF-κB enhancer may be less detrimental in the SupT1 T-cell line that was used in the virus selection experiment because this cell type contains very low levels of this transcription factor (11). To determine whether the NF-κB-to-GABP switch is more detrimental to LTR promoter activity in cells that express higher levels of NF-κB, we also analyzed the promoter constructs in the Jurkat T-cell line. The cells were treated with PMA-PHA at 24 h posttransfection or were left untreated. Compared with the SupT1 cell line, a more dramatic loss of the basal promoter activity was measured in Jurkat cells for the mutant LTR (Fig. 8A), which correlates with the higher NF-κB levels in the latter cell type (11). However, in the presence of either suboptimal amounts of Tat in combination with PMA-PHA (Fig. 8B) or high Tat levels (Fig. 8C), the variant LTR was able to fully overcome this defect. Thus, the variant LTR promoter with a GABP site can outperform the wild-type LTR even in cell types that contain abundant levels of the NF-κB protein. We also analyzed the effect of TNF-α treatment on promoter activity. This proinflammatory cytokine stimulates NF-κB binding activity in the nucleus, but presumably via signaling cascade other than that induced by PMA (52). For a mouse major histocompatibility complex class I gene promoter, it has been reported that tandem NF-κB sites are required for optimal TNF-α induction (23). We therefore compared the wild-type and variant LTR promoters with two (wild type) and one (variant) NF-κB site for their TNF responsiveness in the Jurkat cell line. The results indicate that both types of HIV-1 LTR promoter do not respond to TNF-α treatment (Fig. 8).

We next tested the effects of overexpression of the two GABP subunits on transcription from the wild-type and mutant LTR promoter. SupT1 cells were cotransfected with combinations of LTR-CAT plasmid and Tat, GABPα, and GABPβ1 expression vectors. We plotted the basal LTR activities measured in the absence of Tat (Fig. 9A) and the Tat-activated LTR activities (Fig. 9B). These two values were used to calculate the relative Tat response of the two LTR promoters (Fig. 9C). Cotransfection of the GABP vectors inhibited basal activity of the mutant LTR promoter, whereas the wild-type LTR was relatively unaffected (Fig. 9A). Cotransfection of the mutant LTR-CAT vector with an individual GABP plasmid inhibited transcription approximately twofold, and an eightfold reduction was measured when both GABP plasmids were cotransfected. In contrast, cotransfection of the GABP plasmids in the presence of Tat yielded similar transcriptional activities for the wild-type and variant LTR promoters (Fig. 9B). Thus, overexpression of GABP results in a selective inhibition of the variant LTR promoter, but this GABP-mediated inhibition is fully overcome in the presence of Tat. In other words, the variant promoter is more Tat responsive than the wild-type LTR upon overexpression of GABP (Fig. 9C).

**The GABP site improves virus replication in SupT1 cells.** Interestingly, we observed that the 1-nt deletion in the NF-κB

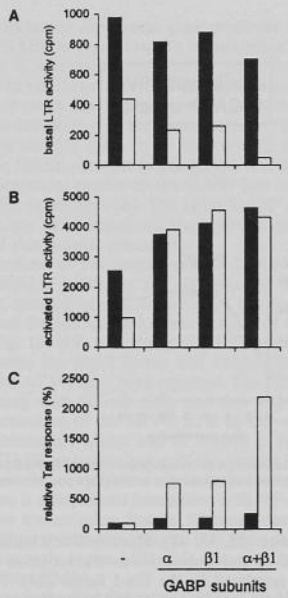


FIG. 9. Overexpression of GABP specifically affects the variant LTR promoter. SupT1 T cells were electroporated with 20 (A) or 1 (B) μg of the wild-type (■) or mutant (□) LTR-CAT in the presence or absence of 2.5 μg of the pcDNA3-Tat expression vector. Vectors encoding the α and β1 subunits of GABP were included individually or in combination as indicated. The relative Tat response (C) was calculated from the data in panels A and B (see Materials and Methods). In this calculation, we corrected for the different amounts of transfected LTR-CAT plasmid. The Tat response of both each promoter in the absence of GABP was set at 100% (in this experiment, an approximately 50-fold induction was measured for both promoters). Similar results were obtained in three independent transfection experiments.

enhancer is also present in the LTR promoter of subtype E viruses (see Discussion). Thus, the NF-κB-to-GABP site modification represents natural variation in the LTR promoter that could provide the different subtype viruses with unique transcriptional properties. To test this, we introduced the 1-nt mutation in the HIV-1 LAI isolate and compared the replication of the wild-type and GABP variant in different cell types, including the SupT1 cell line in which the GABP site was selected. A small but significant increase in replication capacity was observed in these cells (Fig. 10A and B). No difference in replication was measured in other T-cell lines (Fig. 10C and D) and in primary cells (PBMC; Fig. 10E). This selective gain of fitness may correlate with the extremely low amount of NF-κB in SupT1 cells. We also performed a coculture of the two viruses to allow more accurate calculation of the fitness gain. Samples of infected cells were taken over time, and the proviral LTR was analyzed by population-based sequencing to determine the relative concentrations of the wild-type virus and GABP variant (Fig. 11). This experiment demonstrated the rapid outgrowth of the GABP variant. Fitness calculations indicated an approximately 37% increase in replication capacity for the GABP variant compared with wild-type LAI.

## DISCUSSION

We observed a single-nucleotide deletion in the upstream NF-κB site of the tandem enhancer motif in the HIV-1 LTR promoter. This mutation was selected in a long-term culture of a Tat-defective HIV-1 mutant. EMSAs demonstrated loss of NF-κB binding, and a concomitant loss of basal promoter activity was measured. However, outgrowth of this particular LTR variant suggested that the mutation does contribute to improved virus replication. A new binding activity that is largely specific for the mutant LTR was identified in EMSAs with nuclear extracts of unstimulated cells. This DNA-protein complex did not react with NF-κB-specific antibodies, indicating that the factor is not an alternative NF-κB complex. Deletion and mutation analyses delineated the minimal DNA binding site and the sequence-specific core that is recognized by the new protein factor. This DNA sequence is homologous to the binding site recognized by the GABP transcription factor (59), and antibody-mediated supershifts demonstrated that it is indeed GABP that binds to the mutated NF-κB site. The 1-nt deletion decreased binding of NF-κB by a factor 8 and, at the same time, increased the affinity for GABP approximately 14-fold.

The mutant LTR promoter displayed a partial loss of activity at low transcription levels, but this promoter outperformed the wild-type LTR at high transcription levels in the presence of the Tat transactivator protein and PMA-PHA stimulation. A simple interpretation of these results is that NF-κB contributes primarily to basal LTR activity, which is consistent with previous studies (7), whereas the major contribution of the new GABP motif is to improve the level of activated LTR transcription. However, the situation may be more complex. For instance, cotransfection of GABP expression vectors reduced the basal activity of the mutant HIV-1 LTR in a specific manner, suggesting that the GABP site is responsible in part for both the reduced basal promoter activity and increased level of activated promoter activity. It is obvious that such altered promoter characteristics may provide HIV-1 with unique biological properties.

Because the HIV-1 promoter variant was selected in the SupT1 T-cell line, which is known to have an extremely low level of NF-κB protein, we reasoned that the conversion of an NF-κB site into a GABP binding site could represent a SupT1-specific adaptation. In other words, it is possible that this enhancer switch is more detrimental in a cell line with higher NF-κB levels. This was tested in the Jurkat cell line, which expresses high NF-κB levels (11). However, a very similar pattern of reduced basal transcription and improved activated transcription was measured for the mutant LTR promoter in the Jurkat cell line. Obviously, the new LTR enhancer configuration may have been altered to repair, at least partially, the Tat defect of the HIV-1 mutant that was used to initiate the long-term infection experiment. Indeed, the transient transfection data indicate that the mutant LTR with the GABP site is more Tat responsive. In this way, the virus could amplify the residual Tat activity encoded by the mutant Tyr26Ala virus. We measured poor transcriptional activity with the variant LTR promoter in combination with the Tyr26Ala Tat mutant (results not shown). Thus, other changes elsewhere in the HIV-1 genome are likely to have contributed to the reversion event. In fact, a second-site mutation within the *tat* gene was observed for this revertant virus, and this putative Tat revertant protein is currently being analyzed.

It has been reported that other Ets transcription factors also bind the HIV-1 LTR. Multiple weak Ets binding sites were detected in the promoter-distal LTR region, around position

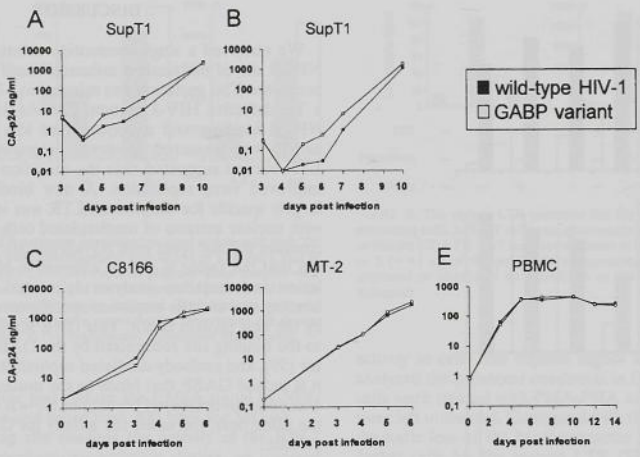


FIG. 10. Replication of wild-type LAI virus and the GABP variant in different cell types. Different T-cell lines (A to D) and primary cells (PBMC; E) were infected with the wild-type and GABP virus (20 [A], 2 [B], 10 [C], 1 [D], and 4 [E] ng of CA-p24). Virus production was measured at several days postinfection.

−145 relative to the transcription start site (4, 22, 53). The Ras/Raf activation pathway, which leads to activation of many transcription factors including Ets proteins, triggers expression from the HIV-1 promoter, and Ras/Raf-responsive elements that overlap the NF-κB elements were identified (6, 9). It has been demonstrated that NF-κB is not responsible for this stimulation, and subsequent analyses revealed the involvement of several Ets transcription factors, including Ets-1, Ets-2, ERGB/Fli-1, and GABP (17, 21, 50). In addition, the HIV-1 sequence overlapping the NF-κB binding sites has been shown to bind other transcription factors such as the zinc finger-containing protein PRDII-BF1 (3) and the cell cycle regulator E2F-1 (32). Except for GABP, we did not test how the single T-deletion within the upstream NF-κB site affects the binding of these factors.

Ets family transcription factors are involved the transcriptional regulation of a variety of viruses (Fig. 12). For instance, the HIV-2 LTR contains a single NF-κB site with an upstream Elf-1 binding site (35, 37, 41). Human T-cell leukemia virus type 1 (HTLV-1) and polyomavirus transcription is, among other factors, regulated by the Ets-1 factor (24). The GABP transcription factor was first identified in studies on transcriptional regulation of the adenovirus E4 promoter (59). A well-known nonviral promoter regulated by GABP is the retino-

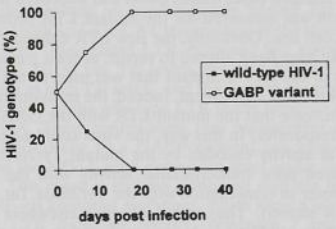


FIG. 11. The GABP variant outgrows the wild-type LAI virus in a mixed infection of SupT1 cells. An equimolar mixture of the wild-type virus and GABP variant (8 ng of CA-p24 each) was used to infect SupT1 cells at day 0. Virus was passaged at the peak of infection onto fresh SupT1 cells. Infected cell samples were used for PCR amplification of a proviral LTR fragment, and population-based sequencing was performed to determine the composition of the virus mixture. Fitness calculations were performed as described previously (20), with the viral generation time set at 2.0 days.

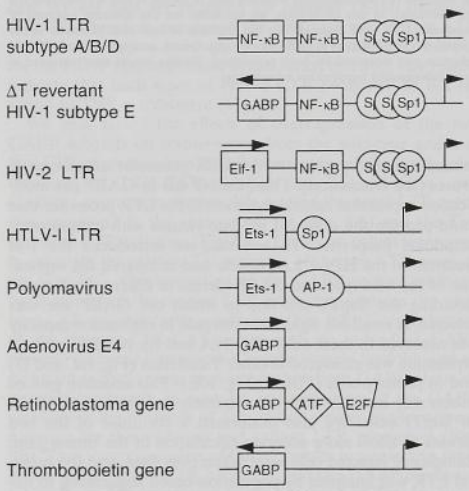


FIG. 12. The GABP site is also present in HIV-1 subtype F. Promoter organizations of several viral and cellular genes with binding sites for Ets factors are shown. The GABP site identified in this study is identical in sequence to the motif present in HIV-1 subtype E. Arrows over the GABP, Ets-1, and Elf-1 motifs indicate the 5′-to-3′ direction of the GGA core recognition sequence. See text for further details.

blastoma (Rb) tumor suppressor gene promoter (46, 51, 55). The role of GABP in regulating Rb transcription may be crucial, since it is speculated that GABP gene inactivation is associated with the occurrence of some malignancies (55). The orientation of most Ets binding sites is such that the core binding sequence GGA is in the coding strand (Fig. 12; arrows over Ets sites indicate the 5′-to-3′ direction of the GGA sequence). The GABP element in the HIV-1 revertant is in the opposite orientation, similar to the GABP site in the thrombopoietin gene promoter (28). The latter GABP element was shown to be the most important transcription factor for the regulation of this cellular promoter.

A characteristic of Ets factors is that they synergize with other transcription factors in the activation of transcription. For instance, interaction of Ets-1 with Sp1 has been proposed to be required for full transcriptional activity of the HTLV-1 promoter (Fig. 12) (19). For the HIV-1 LTR promoter, interactions between the Ets-1 factor and either USF-1 (53) or NF-κB and NFAT (4) have been reported. The Elf-1 factor can interact directly with NF-κB (26), and this may explain the synergistic activation of the HIV-2 LTR by NF-κB and Elf-1 (37). Experiments are under way to analyze in further detail the role of GABP in transcription of the variant HIV-1 LTR, e.g., whether its contribution is orientation specific and whether there is a functional interaction with NF-κB bound at site I or other transcription factors. Although our preliminary protein binding experiments indicate that these factors cannot bind simultaneously to the LTR, this may merely represent a limitation of the EMSA.

There is evidence that the conversion of an NF-κB into a GABP site is not a unique tissue culture adaptation phenomenon. Alignment of the new LTR promoter configuration with published HIV-1 sequences (39–41) revealed identity with LTR sequences of subtype E isolates. Whereas HIV-1 subtypes A, B, and D contain tandem NF-κB sites, subtype E viruses have the typical GABP/NF-κB enhancer configuration as described in this study (Fig. 12). The LTR sequences of a total of 18 subtype E isolates have been determined, and all isolates have the exact GABP site as described and tested in this study (references 39 and 40 and unpublished results from our laboratory). It has been reported that NF-κB binding to the upstream NF-κB site of subtype E viruses is abolished, without an apparent loss of promoter function (40). We can now explain this result: the T-deletion represents a modified rather than a defective enhancer element.

Because HIV-1 subtype E is thought to represent a relatively recent branch of the HIV-1 phylogeny of the current epidemic, it is likely that the T deletion in the upstream NF-κB site occurred at least once during the worldwide spread of HIV-1. We demonstrate that the LAI virus with the GABP site can outgrow the wild-type virus in certain cell types. For animal retroviruses, there is ample evidence for a role of LTR enhancer switches as an important regulator of pathogenicity and oncogenicity (57). The alternative enhancer configuration of the subtype E viruses may also have implications for cell tropism. For instance, it has been reported that HIV-1 subtype E viruses replicate more efficiently than other subtypes in Langerhans cells, the possible target in heterosexual transmission (54), although follow-up studies could not confirm these results (16, 43). The lentivirus equine infectious anemia virus provides an interesting example where the presence of an Ets-1 site in the LTR promoter was found to be essential for productive virus replication in macrophages (10, 38). Despite accumulating sequence data on the genomes of HIV-1 strains belonging to the different subtypes (18, 41), no subtype-specific differences in virus biology have been described. Because it has been suggested that subtype E is a more pathogenic and virulent virus, it will be important to test the biological function of the GABP site-containing LTR promoter in more detail.


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# Chapter 8

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# Genetic Instability of Live, Attenuated Human Immunodeficiency Virus Type 1 Vaccine Strains

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**Live, attenuated viruses have been the most successful vaccines in monkey models of human immunodeficiency virus type 1 (HIV-1) infection. However, there are several safety concerns about using such an anti-HIV vaccine in humans, including reversion of the vaccine strain to virulence and recombination with endogenous retroviral sequences to produce new infectious and potentially pathogenic viruses. Because testing in humans would inevitably carry a substantial risk, we set out to test the genetic stability of multiply deleted HIV constructs in perpetuated tissue culture infections. The Δ3 candidate vaccine strain of HIV-1 contains deletions in the viral long terminal repeat (LTR) promoter and the *vpr* and *nef* genes. This virus replicates with delayed kinetics, but a profound enhancement of virus replication was observed after approximately 2 months of culturing. Analysis of the revertant viral genome indicated that the three introduced deletions were maintained but a 39-nucleotide sequence was inserted in the LTR promoter region. This insert was formed by duplication of the region encoding three binding sites for the Sp1 transcription factor. The duplicated Sp1 region was demonstrated to increase the LTR promoter activity, and a concomitant increase in the virus replication rate was measured. In fact, duplication of the Sp1 sites increased the fitness of the Δ3 virus (Vpr/Nef/U3) to levels higher than that of the singly deleted ΔVpr virus. These results indicate that deleted HIV-1 vaccine strains can evolve into fast-replicating variants by multiplication of remaining sequence motifs, and their safety is therefore not guaranteed. This insight may guide future efforts to develop more stable anti-HIV vaccines.**

Relatively disappointing outcomes have been obtained thus far with a variety of anti-human immunodeficiency virus (HIV) vaccine candidates (41). However, the results of studies with live attenuated simian immunodeficiency viruses (SIVs) in monkey models are promising models for the possible use of live attenuated HIV as a protective vaccine. It has been repeatedly demonstrated that macaques or chimpanzees persistently infected with genetically attenuated, nonpathogenic isolates of SIV or HIV-1, respectively, strongly resist a subsequent challenge with pathogenic virus (2, 12, 36, 45, 47, 53, 57). In addition, there is some evidence that attenuated HIV-1 variants lacking the *nef* gene result in a benign course of infection in humans (16). These results warrant further investigation of this class of anti-HIV vaccines. However, the development of a live attenuated HIV-1 vaccine will face major safety issues, and the question has been raised of how much animal work remains to be done before human vaccine trials can proceed (7, 10, 17, 38). For instance, recent evidence suggests that SIV constructs with multiple gene deletions can be pathogenic in newborn monkeys (3, 58). Another major safety concerns is the fear that the vaccine strain can evolve from an attenuated form to a more virulent, pathogenic form. The latter process of virus evolution can occur either by spontaneous mutation of one of the remaining viral functions or by recombination-mediated repair with parts of the host cell genome, e.g., endogenous retroviral sequences.

In this study, we addressed the genetic stability of multiply deleted HIV-1 variants. To do so, we performed long-term tissue culture infections with the HIV-1 candidate vaccine strains to allow virus evolution to be studied on a laboratory timescale. Evolution of HIV-1 in tissue culture setting has been reported repeatedly. A well-known example of tissue culture evolution occurs during culturing of primary isolates on T-cell lines, which results in the selection of "laboratory-adapted" HIV-1 variants with amino acid changes within the envelope glycoprotein that cause a shift in the host cell range and coreceptor use. Furthermore, many studies with replication-impaired HIV-1 mutants yielded revertant viruses after prolonged in vitro culturing. The analysis of revertant viruses has been used to study interactions within HIV-1 proteins, e.g., the Env glycoprotein (56) and the integrase enzyme (49). This genetic approach has been particularly useful in the dissection of complex RNA motifs, including the TAR hairpin (25, 32), the poly(A) hairpin (5, 14), and the DIS dimerization signal (6). Furthermore, mutations in the DIS RNA motif can be overcome by compensatory mutations within the viral Gag protein, which is most probably due to a direct interaction between the DIS RNA and the Gag protein (35). These combined results demonstrate the enormous genetic flexibility and repair capacity of the HIV-1 retroviral genome.

This genetic flexibility of HIV-1 is likely to be greater in in vivo infections, where a larger number of virion particles are produced. A prominent example is the appearance of drug-resistant HIV-1 variants in patients treated with potent antiviral drugs. For resistance to drugs against the protease enzyme, there is accumulating evidence that the primary resistance mutations within the protease protein cause a partial enzyme defect, which is subsequently restored by secondary mutations in protease and/or compensatory changes within the *gag*-en-

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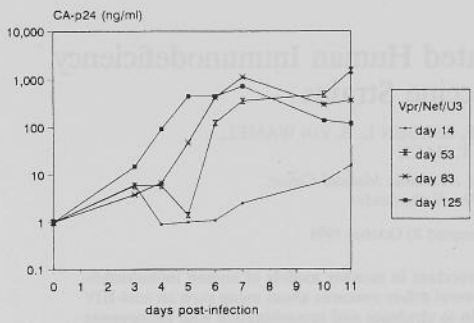


FIG. 1. Improved growth kinetics of Δ3 revertant viruses. The HIV-1 samples represent the Δ3 virus after culturing on SupT1 cells for increasing periods. The perpetuated SupT1 infection was started by electroporation of 10 μg of Δ3 construct, and at the peak of infection, virus was passaged onto fresh, uninfected SupT1 cells. Supernatant samples were taken at several days posttransfection, frozen, and subsequently used to infect SupT1 cells with the same amount of input virus (1 ng CA-p24). Cell samples were also stored for proviral DNA analysis (see Fig. 2).

coded substrate sequences (11, 59). The in vivo capacity for repair of small attenuating gene deletions was also demonstrated by the evolution of an SIV variant with a 12-nucleotide (nt) deletion in the *nef* gene. A sequence duplication event was observed first, and multiple mutations were subsequently selected in the "insert" to create an amino acid sequence that is virtually indistinguishable from that of the wild-type virus (55). Despite these safety concerns, it seems unlikely that HIV-1 can repair large gene deletions. Therefore, whole genes have been deleted in the current live, attenuated vaccine candidates. Nevertheless, it has been reported recently that certain HIV-1 mutants can restore their fitness by second-site changes in unrelated functions encoded by the remaining sequences in the viral genome (15). We therefore tested the genetic stability of the current generation of HIV-1 vaccine candidates in long-term tissue culture infections.

## MATERIALS AND METHODS

**Plasmid constructs and PCR.** The HIV-1 molecular clone used in this study is the chimeric pNL4-3 plasmid (1), which contains the 5′ half of proviral NY5 and the 3′ half of proviral LAV sequences joined at a shared *Eco*RI site in the *vpr* gene. The different deletion mutants were described previously (21), and infectious virus was reconstructed from the 5′ and 3′ half genomes by *Eco*RI digestion and subsequent ligation.

Proviral DNA sequences were PCR amplified across the deletion points. To screen for the *nef*-U3 deletions, we used the primer pair NEF.SEQ and 3′TATA primer (positions 2990 to 3009 and 3756 to 3779 in plasmid p83-10 [21]). The *vif-vpr* region was analyzed by PCR with the primer pair Pol 5′FM and 6N (positions 4932 to 4964 in p83-2 and positions 213 to 237 in p83-10), and the *vpu* region was analyzed with the primer pair Tat-AUG and WS-3 (positions 88 to 107 and 801 to 820 in p83-10). The *nef*-U3 PCR fragment with an insert was introduced in a T/A cloning vector (Promega), and the sequence of multiple clones was analyzed by the *Taq* T7 Dyeprimer cycle-sequencing method (Applied Biosystems) on an Applied Biosystems 373 DNA sequencer.

Long term repeat (LTR)-chloramphenicol acetyltransferase (CAT) reporter constructs with either the wild-type, Δ3 mutant, or 6×Sp1 revertant sequences were constructed as follows. Viral DNA was PCR amplified with the upstream Xho-U3 primer (CCGCTCGAGTGGAAGGGCTAATTCACT), which places an *Xho*I restriction site (underlined) immediately upstream of the U3 region of the LTR, and the downstream U5 primer CN1. This DNA fragment was digested with *Xho*I and *Hin*dIII (position +77 in the R repeat region) and inserted into *Xho*I-*Hin*dIII-cleaved pBlue-3′LTR-CAT (33). The Tat expression vector pcDNA3-Tat and the CAT assay method were described previously (54).

The 6×Sp1 revertant sequence was introduced into the *nef*-U3 and Δ3 viruses as follows. First, the 6×Sp1 region was cloned into the 3′ LTR of the *nef*-U3-deleted 3′-half plasmid (p210-8 in reference 21) by replacement of a 216-bp 3×Sp1 fragment by a 255-bp 6×Sp1 fragment via *Eco*RV and *Bfr*I restriction sites in the U3-R region. Infectious virus was obtained by ligation of this 3′-half plasmid (*nef*-U3-6×Sp1) with a 5′-half plasmid (either the wild type or the Vpr deletion variant) as described above. Virus stocks were produced in SupT1 cells.

**Cells and viruses.** SupT1 cells were grown in RPMI medium containing 10% fetal calf serum and penicillin-streptomycin. Transfection of SupT1 cells by electroporation was carried out as described previously (13) with ligation mixtures that contain a total of 10 μg of DNA. Culture supernatants containing infectious virus were harvested at the peak of infection and stored in small aliquots at −70°C. These virus stocks were carefully quantitated by CA-p24 enzyme-linked immunosorbent assay (4) and used in infections of SupT1 cells. Infections were performed in 1.5 ml of RPMI medium containing $3 \times 10^5$ cells, and virus production was monitored by measuring CA-p24 antigen production. Donor peripheral blood mononuclear cells (PBMC) were prepared, cultured, and infected with HIV-1 as previously described (4).

Long-term culturing to select for revertant viruses was initiated by massive transfection of SupT1 cells (the ligation mixture containing 5 μg of both the 5′ and 3′ genome fragments was electroporated in $5 \times 10^6$ cells). In the first weeks, the cells were split when necessary. As soon as virus spread was apparent, as indicated by the presence of syncytia, the virus-containing culture supernatant was passaged onto uninfected SupT1 cells, initially with large samples of up to 1 ml and later with much smaller samples, as described previously (14).

Direct competition experiments with two virus variants were performed as described previously (34). We used equal amounts of the two viruses based on the CA-p24 measurements. The compositions of the 3×Sp1 and 6×Sp1 virus

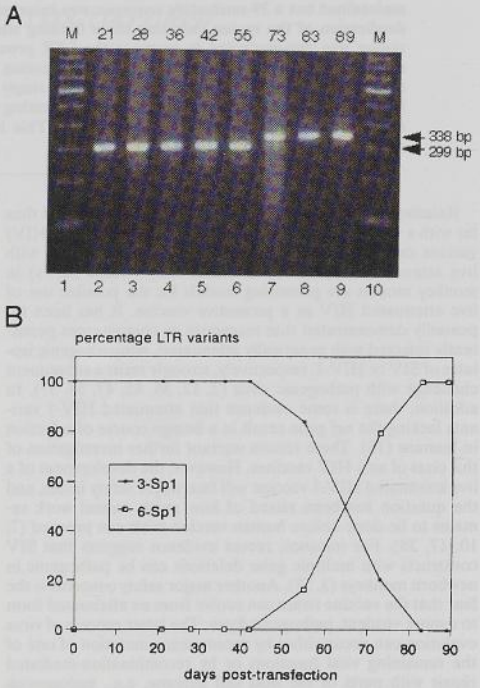


FIG. 2. The Δ3 mutant creates an LTR promoter with six Sp1 sites. (A) Cell samples taken on days 21, 28, 36, 42, 55, 73, 83, and 89 of the perpetuated SupT1 infection were used to extract cellular DNA as described previously (14), and the *nef* LTR region of the HIV-1 genome was PCR amplified. A 299-bp fragment is produced with the Δ3 mutant template (three Sp1 sites), and a revertant fragment of 338 bp is observed (six Sp1 sites). The day of cell harvest is indicated at the top of the gel. A 100-bp DNA ladder is provided in lanes 1 and 10 (lanes labeled M). (B) Quantitation of the ethidium bromide-stained gel was performed with the Kodak digital science 1D system and used to calculate the fractions of Δ3 mutant (three Sp1 sites) and Δ3 revertant (six Sp1 sites).

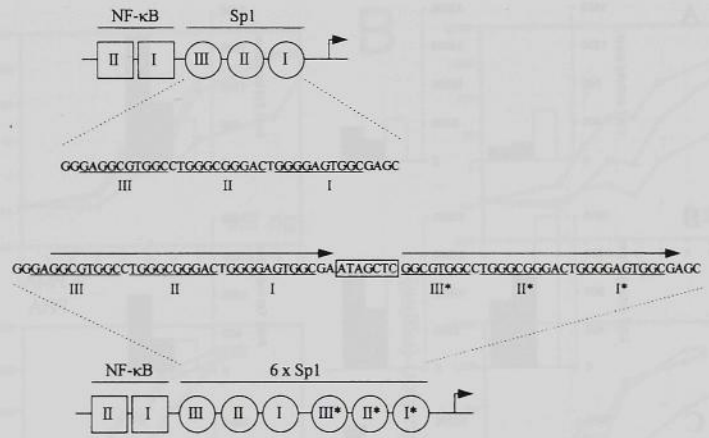


FIG. 3. Duplication of the three Sp1 sites in the LTR promoter. The wild-type LTR promoter contains two NF-κB sites (squares) at positions −105 to −96 and −91 to −82 relative to the RNA start site at +1 (arrow) and three Sp1 sites (circles) at positions −78 to −69, −67 to −58, and −56 to −47. The Δ3 LTR carries a deletion of the upstream part of the U3 promoter region (starting at position −150). The nucleotide sequence of the three Sp1 sites is shown, with the 10-mer binding sites underlined. The lower panel shows the Δ3 revertant with the 39-nt insert. The insert consists of a 32-nt duplication (arrows) and a 7-nt sequence of unknown origin (boxed). Of the three new Sp1 motifs, the upstream site III* is a partial copy of site III, and it is therefore unknown whether site III* can bind the Sp1 factor.

mixtures were analyzed by PCR amplification of the proviral genomes to monitor the LTR length polymorphism. These data were used to calculate the relative fitness as described previously (26). In brief, the relative fitness of the 6×Sp1 virus was approximated from the equation $p/q = [p(0)/q(0)] \times (\text{fitness})^T$, where $p$ is the proportion of 6×Sp1 virus, $q$ is the proportion of 3×Sp1 virus, 0 indicates time zero, and $T$ is the time in viral generations (2 days per generation).

## RESULTS

We analyzed the in vitro replication capacity of a set of 20 HIV-1 variants with single or multiple deletions of accessory genes (*vif*, *vpr*, *vpu*, and *nef*) and of the upstream part of the U3 promoter region (21). Most variants with multiple deletions were severely replication impaired in primary human lymphocytes, e.g., *vpr-nef*-U3 (Δ3), *vpr-vpu-nef*, and all viruses with the *vif* deletion (data not shown; see also reference 21). The evolutionary capacity of such crippled viruses will be severely limited because the generation of new variants depends on random mutations introduced during viral replication by the error-prone reverse transcriptase. Therefore, we tested the deleted HIV-1 strains in a human T-cell line in which the defects caused by inactivation of some of the HIV-1 accessory genes are less pronounced (51). For instance, the Δ3 vaccine strain replicated with delayed kinetics in the SupT1 T-cell line but was eventually able to produce massively infected cultures as measured by the CA-p24 levels in the culture supernatant and the appearance of syncytia (data not shown). This optimized culture system was used for the long-term evolution studies of several HIV-1 constructs with multiple deletions. Virus was passaged onto uninfected cells for 4 months to select for faster-replicating revertants, and increased virus replication was noticed in several cultures. To accurately compare the replication kinetics of viruses sampled over time, we used identical amounts of these virus samples to infect SupT1 cells. The results obtained with the samples of the Δ3 virus are shown in Fig. 1 and indicate that replication improved dramatically after approximately 2 months of culturing. A similar gain of replication capacity was observed with other HIV-1 deletion constructs (e.g., vif-vpu and *vif-nef*-U3) but not with all variants.

One possibility for repair of deleted gene functions is the insertion of a cellular gene with a similar function through recombination. To check whether the deletions in the HIV-1 genome were maintained, we performed PCR analyses across the deletion sites for all viruses that were cultured for 4 months. No insertions were observed for any of the viruses samples, except for the Δ3 virus, in which a large insert appeared over time in the *nef*-LTR region. To analyze this process in more detail, SupT1 cell samples taken at different times were used to extract genomic DNA and to amplify the *nef*-LTR region of integrated proviruses (Fig. 2A). The 299-bp fragment predicted for the Δ3 virus was observed in the first 2 months of culturing, but a larger fragment appeared around day 55. This new fragment became the most prominent band at day 73 and completely replaced the original fragment at later times. Quantitation of the data indicated that the size variant was able to efficiently outgrow the Δ3 virus, with an increase in relative concentration from 16 to 80% in only 18 days (Fig. 2B).

The complete Nef-LTR region of the Δ3 revertant was sequenced (Fig. 3). Interestingly, both deletions in the Nef and U3 region were still present, but a 39-nt fragment was inserted in the promoter region containing the Sp1 sites that bind the constitutively expressed Sp1 transcription factor (31). The insert consists of a 32-nt duplication and a 7-nt sequence of unknown identity. It seems likely that the Sp1 region was duplicated during reverse transcription of the viral genome. Tandem repeat sequences such as the Sp1 sites are known to be subject to deletion or duplication by a slippage-realignment mechanism (42). Inspection of the nucleotide sequence of the insert clearly indicates that the whole Sp1 region was copied in a single step. The alternative, i.e., multiple rounds of duplication of a single Sp1 site, can be excluded also because no PCR products of intermediate length were observed in the evolution experiment (Fig. 2A). Thus, a novel LTR promoter configura-

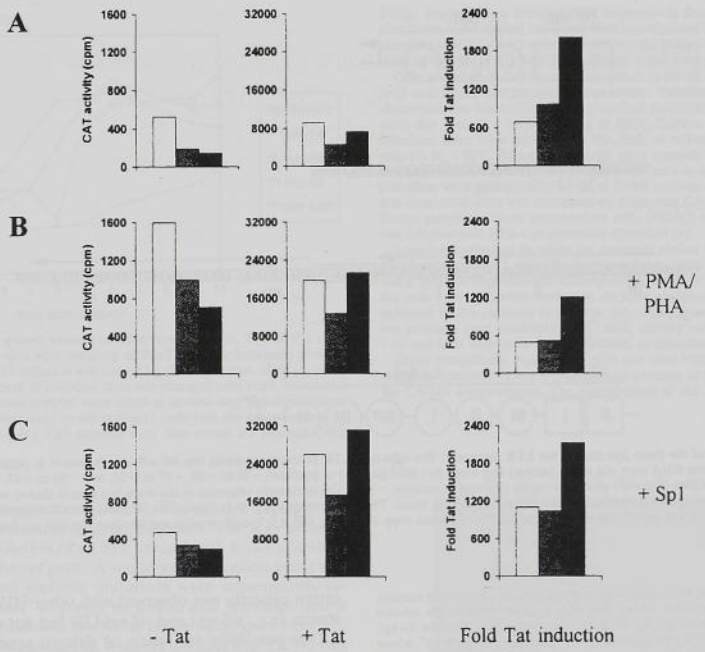


FIG. 4. The Δ3 LTR promoter gains activity by duplication of the Sp1 sites. (A) SupT1 T cells ($5 \times 10^6$) were electroporated with 40 μg of LTR-CAT reporter construct (wild type, Δ3 mutant, and Δ3 revertant) in the absence of Tat (left) or with 1 μg of LTR-CAT plasmid in combination with 2.5 μg of pcDNA3-Tat (middle). The cultures were harvested on day 3 for CAT assays. The fold Tat-mediated activation of LTR-transcription was calculated and is plotted (right). (B and C) Parallel transfections were performed on cells that were treated with PMA-PHA (final concentrations 25 ng/ml and 1 μg/ml, respectively) on day 1 posttransfection (B), and transfections were repeated in the presence of an Sp1 expression plasmid (C). A representative experiment is shown, and similar results were obtained in four independent transfections. Furthermore, similar results were obtained in transfections with other cell types, including non-T cells. The basal and Tat-induced promoter activities cannot be compared directly because different amounts of LTR-CAT plasmid were used. When the results were corrected for this difference, an approximately 200-fold induction of LTR activity was measured.

tion consisting of two NF-κB and six Sp1 binding sites was created during replication of the Δ3 virus.

To test whether duplication of the three Sp1 motifs improved the transcriptional activity of the Δ3 promoter, we constructed a set of LTR-CAT reporter plasmids, including the wild-type LTR promoter, the Δ3 mutant lacking the upstream part of the U3 region, and the Δ3 revertant with six instead of three Sp1 sites. These plasmids were transfected into SupT1 cells in the presence or absence of a second plasmid encoding the viral Tat *trans*-activator protein. The results of a representative experiment are shown in Fig. 4A (left and middle), and the fold transcriptional activation was calculated (right). To boost the low level of basal LTR transcription, similar transfections were performed in cell cultures that were activated with phorbol myristate acetate and phyrohemagglutinin (PMA-PHA) on day 1 posttransfection (Fig. 4B) and in the presence of additional Sp1 encoded by an expression plasmid (Fig. 4C). Comparison of the wild-type and Δ3 promoters indicated an approximately twofold reduction of transcriptional activity upon deletion of the upstream U3 sequences, in both the absence and presence of Tat. A further reduction of the LTR activity in the absence of Tat was measured for the Δ3 revertant with six Sp1 sites. However, Tat-activated transcription of the Δ3 revertant was improved relative to that of the Δ3 mutant, and an expression level comparable to that of the wild-type LTR was reached. Similar results were obtained in cells activated with PMA-PHA and upon overexpression of Sp1. Our finding that the six Sp1 sites are beneficial only in the presence of Tat is consistent with the proposed functional interaction between the Tat and Sp1 proteins during LTR-mediated transcription (30, 39, 48).

Enhanced LTR promoter activity of the 6×Sp1 variant is consistent with the improved replication of the Δ3 revertant virus, but it cannot be excluded that other genomic changes contribute to the reversion phenotype. To unequivocally prove that the duplication of the Sp1 region is responsible for the observed fitness gain, we introduced the 6×Sp1 sites in the 3′ LTR region of two molecular clones, the *nef*-U3 plasmid and the *vpr-nef*-U3 (Δ3) variant. Virus stocks were produced and used to infect SupT1 cells. We used equal amounts (based on CA-p24) of the 6×Sp1 viruses and the appropriate control viruses (Fig. 5). The contribution of the six Sp1 sites in the *nef*-U3 background is shown in Fig. 5A for three infections with a variable amount of input virus (top, 0.2 ng; middle, 1.0 ng; bottom, 5.0 ng). Introduction of the six Sp1 sites improved the replication of the *nef*-U3 virus to a level very similar to that of the wild-type control. The increase in virus replication capacity is even more prominent in the Δ3 background (Fig. 5B).

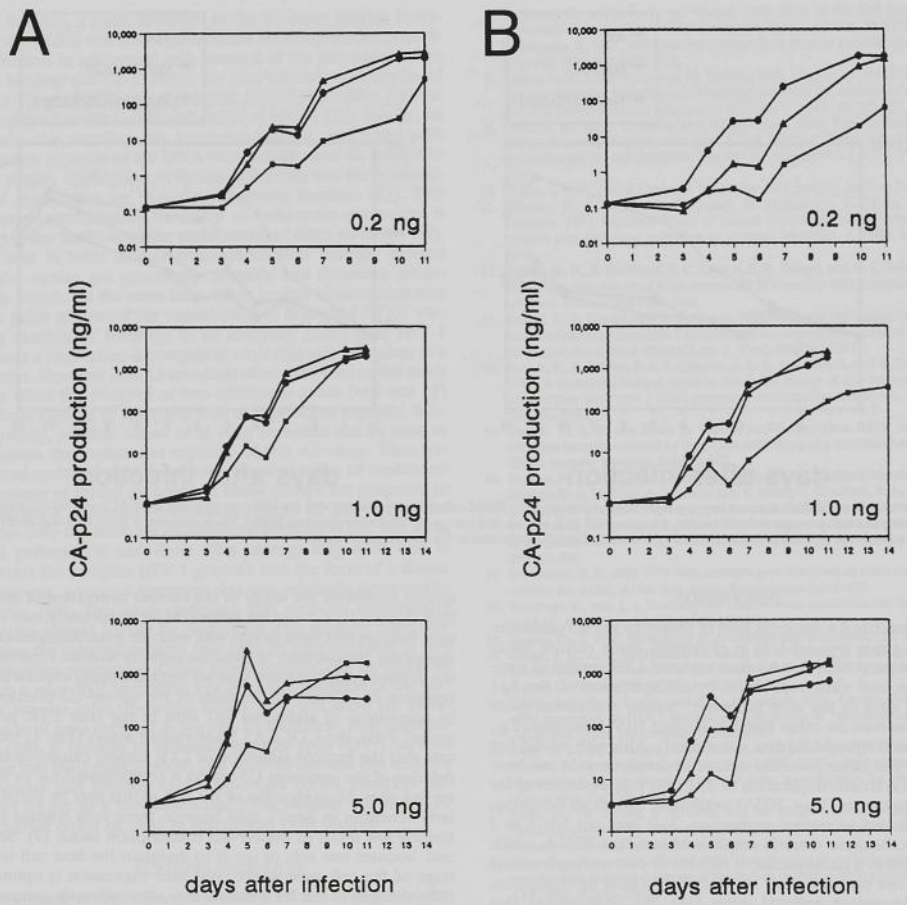


FIG. 5. The reconstituted 6×Sp1 virus replicates with wild-type kinetics. Virus production in SupT1 cultures after infection with wild-type virus (▲ and the *nef*-U3 (■) and *nef*-U3-6×Sp1 (●) variants (A) and the *vpr* single-deletion mutant (▲), the *vpr-nef*-U3 (Δ3) mutant (■), and the *vpr-nef*-U3-6×Sp1 revertant (●) (B). The infections were performed in triplicate with different amounts of input virus: 0.2 ng of CA-p24 (top), 1.0 ng of CA-p24 (middle), and 5.0 ng of CA-p24 (bottom). Virus replication was monitored by measuring CA-p24 production in the culture supernatant. The cultures were split 1:5 at several times postinfection to sustain cell growth and virus replication; this resulted in small decreases in CA-p24 values.

In fact, the Δ3 virus with six Sp1 sites replicated faster than did the singly deleted *vpr* virus, which was included as a control. We also performed mixed-infection experiments with pairs of viruses to demonstrate the gain of fitness by duplication of the Sp1 region. Infections were initiated with equal amounts of the 6×Sp1 virus and the 3×Sp1 control, proviral samples were analyzed over time by LTR PCR amplification, and the composition of the viral mixture was determined by size separation of the LTR fragments on a gel as in Fig. 2 (data not shown). Rapid outgrowth of the 6×Sp1 variant was observed in both the *nef*-U3 and *vpr-nef*-U3 (Δ3) contexts. Based on the results of this internally controlled competition experiment, we calculated a relative gain of virus fitness of 30 and 60%, respectively.

There is abundant evidence that certain LTR promoter-enhancer motifs can affect virus replication in a cell-type-specific manner (8, 9, 29, 40, 43). Although we showed a gain of fitness of the 6×Sp1 variant virus in the SupT1 cell line that was used for the evolution experiment, we also wanted to know whether this promoter adaptation is beneficial in primary cells. PBMC were infected with equal amounts (10 ng of CA-p24) of the 6×Sp1 viruses (in both the *nef*-U3 and Δ3 backgrounds) and the appropriate control viruses (Fig. 6). Interestingly, the 6×Sp1 sites did not significantly improve replication in the *nef*-U3 background (Fig. 6, left), and a small negative effect was measured in the Δ3 context. This effect was verified in more sensitive competition experiments (data not shown).

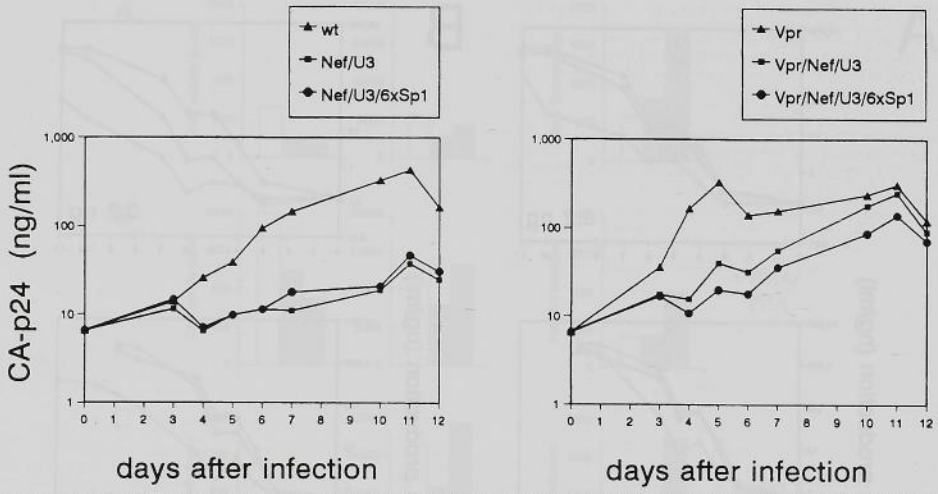


FIG. 6. The 6×Sp1 variant does not improve replication in primary cells. PBMC cultures were infected with the wild-type virus and the *nef*-U3 and *nef*-U3-6×Sp1 variants (left) and the *vpr* single deletion mutant, the *vpr-nef*-U3 (Δ3) mutant, and the *vpr-nef*-U3-6×Sp1 revertant (right). Equal amounts of input virus was used (10 ng of CA-p24). Virus replication was monitored by measuring CA-p24 production in the culture supernatant.

## DISCUSSION

We described a dramatic gain of fitness by the Δ3 candidate vaccine strain (*vpr-nef*-U3) in prolonged tissue culture infections. In particular, this Δ3 virus restored LTR-mediated transcription and virus replication by multiplication of the Sp1 binding sites in the core promoter. Similar replication gains were observed for other multiply deleted HIV-1 variants (e.g., *vif-vpu* and *vif-nef*-U3 [data not shown]). Although we did not analyze the latter revertant viruses in detail, they do not have the characteristic duplication of Sp1 sites that we observed for the Δ3 revertant. Thus, HIV-1 exhibits an enormous evolutionary potential to restore replication. This may not come as a surprise, because there is ample evidence that HIV-1, which replicates as a quasispecies, is capable of overcoming a variety of selective pressures that are intended to limit its replication, including potent antiviral drugs (18). We demonstrate that replication-impaired HIV-1 variants with multiple gene deletions can improve their fitness within a relatively short culture period in an optimized in vitro system. We are obviously unable to directly translate the evolutionary potential of the Δ3 virus as measured in tissue culture to HIV-1 infections in humans. In fact, we found that this particular LTR modification does not improve virus replication in primary cells, suggesting that we may have selected for a SupT1-specific promoter change. Other LTR promoter motifs have also been demonstrated to function in a cell-type-specific manner (9, 40, 43). Nevertheless, because many more viruses are usually replicating in the in vivo situation, it seems unavoidable that other escape routes will be found in vivo.

Although the precise correlation between replication and pathogenicity is unknown, it is likely that a virus revertant with improved fitness will also regain pathogenic potential. For instance, the *vpr-nef*-U3-6×Sp1 revertant replicates more efficiently than does the singly deleted *vpr* virus in SupT1 cells, and an SIV variant with a single Vpr gene deletion induces AIDS in rhesus monkeys (20, 28). These combined results cast serious doubts on the safety of the current generation of multiply deleted HIV-1 vaccine strains. Most importantly, our results indicate that virus strains with multiple gene deletions can apparently restore their replication capacity without repairing the deleted gene functions. Can we mechanistically explain the reversion of a virus with deletions of the *vpr-nef*-U3 functions by acquisition of additional Sp1 sites in the core LTR promoter? First, the LTR-CAT transcription assays (Fig. 4) indicate that the twofold inhibition of LTR activity caused by the deletion of the upstream U3 region is compensated for by the six Sp1 sites. Multiplication of the Sp1 motifs may be particularly beneficial in SupT1 cells because these cells contain extremely low levels of the NF-κB transcription factor (9). Second, because one role of *vpr* is to maintain the host cell in a stage of the cell cycle where viral gene expression is optimal (22), changes in the LTR motifs may also indirectly compensate for a *vpr* defect. Consistent with this idea, expansion of the Sp1 region caused a more dramatic gain of fitness in the *vpr-nef*-U3 mutant than in the *nef*-U3 mutant in SupT1 cells (Fig. 5). Thus, part of the reversion is likely to occur at the level of viral gene expression. The Δ3 revertant with six Sp1 sites does not fully regain the wild-type fitness, which may be due in part to the inability to rescue the deleted Nef function.

There is some precedent for variation in the number of Sp1 binding sites in the LTR promoter of HIV-1. Several HIV-infected individuals were found to contain isolates with four Sp1 sites (34), and one natural isolate with five Sp1 sites was recently identified (44). It was shown that introduction of a fourth Sp1 site has a small but significant effect on LTR transcription and virus replication in SupT1 cells (34). Multiple Sp1 binding sites are found in various viral and cellular promoters, but the number of motifs varies widely, with up to eight sites in the cardiac α-actin gene (24). There is ample evidence for changes in host cell tropism or modulation of the viral oncogenic or pathogenic properties by variation in LTR promoter motifs in animal retroviruses (reviewed in reference 52).

For instance, a point mutation in the Moloney murine leukemia virus LTR was shown to increase transcription and enable replication in embryonal cells because of the generation of an Sp1 binding site (23). There are also numerous examples of more blatant LTR rearrangements, including deletion and duplication of motifs in different clades of HIV-1 (37). Finally, we should also mention the reversion analysis performed with enhancer mutants of the DNA virus simian virus 40. Similar to our results, duplication of existing elements was the predominant mechanism for regaining promoter function (27). This apparent evolutionary flexibility of eukaryotic promoters is largely due to the modular architecture of these elements (19).

These in vitro studies demonstrate that multiply deleted HIV-1 strains are genetically unstable and therefore potentially unsafe. At the same time, these in vitro observations may also guide us toward the construction of improved HIV-1 vaccine candidates. Removal of all accessory genes from HIV-1 creates a replication-incompetent virus that will be useless as a vaccine. However, the Δ3 revertant virus described in this study may allow the removal of two additional genes (*vpu* and *vif*) without leading to complete loss of replication capacity. Subsequently, another round of in vitro evolution can be used to optimize the replication capacity of this Δ5 virus. Thus, repeated cycles of gene deletion and optimization of replication by means of forced evolution in tissue culture are proposed to generate a replication-competent version of HIV-1 that encodes only the basic set of retroviral proteins (Gag, Pol, Env, and perhaps Tat and Rev). This strategy may allow one to convert the complex HIV-1 genome into the form of a simple retrovirus, a vaccine approach that was originally proposed by Temin (50). This study indicates that such an evolutionary strategy should ideally be performed with primary cells, otherwise the selected variants may have an unpredictable in vivo replication phenotype. Other safety features can be added to such a mini-HIV backbone. For instance, insertion of the herpes simplex virus thymidine kinase gene will allow the elimination of cells carrying proviruses by treatment with ganciclovir (46). It remains to be tested whether such HIV-1 variants have lost their pathogenicity and whether humans can mount a response that protects against wild-type HIV-1 infection. Besides its use as a live, attenuated virus vaccine, the mini-HIV construct could be used as inactivated virus vaccine.

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# Summary

# Summary

We studied the structure and function of the Tat transcriptional activator protein of the human immunodeficiency virus type 1 (HIV-1) and the regulation of transcription from the LTR promoter. In **Chapter 1**, a brief, molecular overview of HIV-1 replication is presented, with an emphasis on the regulation of viral transcription by the Tat protein. This small protein is essential for virus replication and activates transcription from the viral Long Terminal Repeat (LTR) promoter through a unique mechanism. The mode of action of Tat involves binding to an RNA target in the nascent transcript (the TAR RNA hairpin) and interaction with cellular kinases (e.g. the cyclinT/cdk9 complex) that subsequently phosphorylate and activate the RNA polymerase II complex. Despite intensive research over the past ten years, little is known on the structure of the Tat protein, which is largely due to the poor solubility of Tat and ready oxidation of its cysteine-rich domain. In the work described in this thesis, we have used a combination of biochemical and virological methods to study the HIV-1 LTR promoter, including the TAR RNA element, and in particular the transcriptional function of mutant Tat proteins and the replication characteristics of Tat-mutated viruses.

**Chapter 2** focusses on the precise structure requirements for the transactivation function of the TAR RNA hairpin motif. Although the binding site for the Tat protein is formed by the upper half of this hairpin, including the three nucleotide bulge, it was found that the full-length stem of the TAR hairpin structure is required for optimal transcriptional activation.

It has been reported that Tat produced by HIV-1 infected cells can have an effect on bystander cells, e.g. resulting in the activation of certain cellular genes. In **Chapter 3**, we analyzed the mechanism of this *inter*-cellular Tat effect, and concluded that the Tat protein itself is transported from one cell to the nucleus of the other cell. We also demonstrated that the C-terminal domain of Tat, containing an RGD motif that has been suggested to interact with integrins on the cell surface, is not involved in this process.

One cellular gene whose expression has been reported to be negatively affected by Tat is MHC class 1. Expression of this gene is important for the recognition of virus-infected cells by the immune system, and downregulation by Tat may provide HIV with a means to escape immunesurveillance. This repression has been proposed to occur at the transcriptional level by the C-terminal Tat domain (amino acids 73 to 86). We studied this mechanism in **Chapter 4** with a mutant virus that expresses only a short 71 amino acid Tat form that is encoded by the first *tat* exon. Although a significant contribution of the C-terminal Tat domain to LTR transactivation and

virus replication was measured, our results could not confirm the idea that this Tat domain is critical for MHC class 1 downregulation.

To determine the relationship between Tat activity and virus replication, we constructed a large set of Tat mutants. This set was tested in transfection assays with an LTR-CAT reporter construct, as well as in the context of the replicating virus.

From these assays, we concluded in **Chapter 5** that the activity of Tat, as measured in LTR-CAT assays, directly parallels the replication capacity of the corresponding virus mutants. It was also noticed that a small reduction in Tat activity already affects viral replication, indicating that the Tat is indeed esential for optimal viral replication. We emasured that a threshold level of around 15% Tat activity is required for virus spread in tissue culture.

With several of the replication-impaired HIV-1 Tat mutants described in chapter 5, we performed long-term virus cultures to select for faster replicating variants with improved Tat function. The rationale behind this genetic approach is that compensatory second site mutations in Tat may provide details on intramolecular contacts in the tertiary structure of this protein. In **Chapter 6**, we describe the analysis of a large number of such viruses that regained rapid replication potential over time. We obtained one revertant virus that had acquired a mutation at a second site in the *tat* gene while retaining the original inactivating mutation. This second-site mutation partially restores transactivation activity and virus replication, but is not specifically associated with the original mutation, since it can also improve the activity of the wild-type Tat protein. Despite the large number of revertant viruses, little information on the tertiary fold of the Tat protein was obtained due to the lack of compensatory second-site mutations observed in the *tat* gene. This genetic approach to study protein structure may not be a good method to analyze the tertiary fold of small proteins.

Since the revertant viruses obviously had regained replication capacity but most of them did not improve the Tat function, we analyzed the LTR promoter region to search for compensatory mutations that may render HIV-1 transcription less dependent on the activity of the Tat transactivator protein. An intriguing mutation was observed in one revertant virus, where an NF-kB enhancer element was inactivated by a single nucleotide deletion (**Chapter 7**). Further analysis revealed that this modified sequence was able to bind a novel transcription factor of the Ets family, yielding a virus with different promoter and replication characteristics. Interestingly, an identical mutation is present in the LTR of HIV-1 subtype E viruses, suggesting that the presence of this modified enhancer element has relevance for the biology of this clade of the HIV-1 epidemic.

In **Chapter 8** we analyze the genetic stability of a potential HIV-1 vaccine strain with large deletions in the *vpr* and *nef* genes and in the U3 upstream enhancer of the LTR promoter. This virus replicates with an attenuated phenotype in transformed and primary cell types, and the SIV equivalent has been used effectively as a live-attenuated virus vaccine. It is demonstrated that this virus also gains replication capacity upon prolonged *in vitro* culturing, and analysis of the fast-replicating revertant revealed a 39 nucleotide insertion in the LTR promotor. Specifically, a duplication of the three binding sites for the Sp1 transcription factor was observed. This altered promoter configuration enhances the transcriptional activity of the LTR and profoundly increases the replication capacity of the vaccine strain. This study indicates that live-attenuated strains of HIV-1 with multiple gene deletions are genetically unstable and therefore unsafe for vaccine trails in human beings.

# Samenvatting

# Samenvatting

Dit proefschrift beschrijft een studie naar de regulatie van transcriptie van de Humaan Immuundeficiëntie Virus type 1 (HIV-1) LTR promoter en naar de structuur en functie van het Tat transactivatie eiwit.

In **Hoofdstuk 1** wordt een beknopt overzicht van de moleculaire biologie van dit virus gegeven, met de nadruk op de transcriptionele regulatie door het virale Tat eiwit. Dit kleine eiwit is essentieel voor virusreplicatie en transactiveert de virale promoter, gelegen in de Long Terminal Repeat (LTR). Het mechanisme van Tat transactivatie omvat binding van het eiwit aan een haarspeld structuur (TAR), die gevormd wordt door het 5' uiteinde van het virale RNA. Met deze interactie brengt Tat een kinase in de nabijheid van de promoter en dit complex phosphoryleert en activeert het RNA polymerase II enzym. Er is nog weinig bekend over de structuur van het Tat eiwit en biofysische studies hiernaar worden bemoeilijkt doordat het eiwit makkelijk oxideert en slecht oplosbaar is. In dit proefschrift wordt met behulp van biochemische en virologische methoden getracht inzicht te krijgen in de regulatie van de activiteit van de virale promoter, waartoe ook het TAR RNA element behoort. De nadruk ligt met name op de bestudering van mutant Tat eiwitten en de replicatie van virussen met een mutant *tat* gen.

In **Hoofdstuk 2** wordt een verband gelegd tussen de stabiliteit van de TAR RNA haarspeld en haar functie in het activatieproces van de LTR promoter. Hoewel de bindingsplaats voor het Tat eiwit wordt gevormd door de bovenste helft van de TAR RNA haarspeld, blijkt dat voor optimale activatie door Tat ook de onderkant van TAR een gepaarde structuur dient aan te nemen.

Eerdere studies rapporteerden dat Tat, geproduceerd door HIV-1 geinfecteerde cellen, een effect kan hebben op ongeinfecteerde, omliggende cellen, bijvoorbeeld in de expressie-regulatie van een aantal cellulaire genen. **Hoofdstuk 3** beschrijft een studie naar het mechanisme van dit intercellulaire Tat effect. Wij concluderen dat het Tat eiwit zelf door de ene cel uitgescheiden en door de andere opgenomen en naar de kern getransporteerd wordt. Tevens tonen wij aan dat een RGD eiwitmotief in het C-terminale domein van Tat, waarvan gesuggereerd is dat het een interactie aangaat met integrines op het celoppervlak, niet verantwoordelijk is voor de opname door cellen.

Eén cellulair gen waarvan is gesuggereerd dat haar expressie negatief gereguleerd wordt door Tat is MHC klasse 1. Expressie van dit gen is essentieel voor herkenning van geïnfecteerde cellen door het immuunsysteem, en remming hiervan zou het virus een mogelijkheid bieden om te ontsnappen aan vernietiging. Deze repressie door het C-terminale domein van Tat (aminozuren 73 - 86) ligt op het niveau van de transcriptie. Dit repressie-mechanisme werd bestudeerd in

**Hoofdstuk 4** door een mutant *tat* gen te construeren dat alleen een 71 aminozuur Tat vorm, dat gecodeerd wordt door het eerste *tat* exon, tot expressie brengt. Hoewel de 14 C-terminale aminozuren van het wild-type Tat eiwit een significante bijdrage leveren aan transactivatie van de virale promoter en virus replicatie, kon het negatieve effect van dit eiwit domein op de regulatie van MHC klasse 1 genexpressie niet worden aangetoond.

**Hoofdstuk 5** beschrijft de constructie van een grote set Tat mutanten die gebruikt werd om de relatie tussen Tat activiteit en virus replicatie te bestuderen. Hiertoe werden deze mutanten getest in transiente transfectie experimenten met een LTR-CAT reporter plasmide en in de context van het replicerende virus. Geconcludeerd werd dat activiteit van Tat mutanten en de replicatie van de corresponderende virus mutanten op een lineaire manier aan elkaar gerelateerd zijn. Een kleine reductie in Tat activiteit heeft reeds verminderde virus replicatie ten gevolge, wat aangeeft dat Tat functie essentieel is voor optimale replicatie van HIV-1. Verder werd een drempelwaarde van 15% Tat activiteit voor productieve virus replicatie gemeten.

Enkele van de Tat mutant virussen met een replicatie-defect die zijn beschreven in Hoofdstuk 5 werden gebruikt in lange-termijn virus kweken om te selecteren voor snel-groeiende virussen met een verbeterde Tat functie. De gedachte achter deze aanpak is dat zulke revertant virussen mogelijk compenserende mutaties bevatten op secundaire plaatsen in het *tat* gen, die informatie kunnen opleveren over intramoleculaire contacten in de driedimensionale vouwing van het Tat eiwit. In **Hoofdstuk 6** wordt het *tat* gen van een groot aantal revertant virussen geanalyseerd op de aanwezigheid van mutaties die de Tat functie verbeteren. Op deze manier identificeerden we een Tat variant met een mutatie op een secundaire positie in het *tat* gen, waarin de primaire, inactiverende mutatie onveranderd aanwezig bleek. Deze zogenaamde 'second site' mutatie was in staat zowel de activiteit van het mutant eiwit als virus replicatie te verbeteren, maar bleek echter niet specifiek geassocieerd met de originele mutatie, omdat na introductie van deze mutatie in het wild-type *tat* gen ook de activiteit van dit eiwit verhoogd werd. Ondanks het grote aantal virus revertanten leverde deze analyse, door een gebrek aan 'second-site' revertanten, weinig informatie op omtrent de tertiare structuur van het Tat eiwit.

Aangezien de virus revertanten goed repliceerden, maar in de meeste gevallen de Tat functie niet was verbeterd, werd de LTR promoter bekeken op de aanwezigheid van compenserende mutaties, die het virus mogelijk minder afhankelijk maken van een goed functionerend Tat eiwit. In **Hoofdstuk 7** wordt voor één revertant virus een belangwekkende mutatie in een van de twee NF-κB bindingsplaatsen in de promoter beschreven, waar door deletie van één nucleotide de interactie van NF-κB met het DNA verstoord bleek. Verdere analyse bracht aan het licht dat door deze deletie nu een bindingsplaats was gecreëerd voor een transcriptie factor

uit de Ets familie. Deze alternative promoterconfiguratie vertoonde een karakteristiek transcriptiepatroon in transfectie experimenten, en verbeterde virus replicatie in sommige celtypen. Een identieke mutatie is aanwezig in de LTR van natuurlijke virus isolaten van HIV-1 subtype E, hetgeen suggereert dat deze variant promoter ook relevant is voor de biologie van deze tak van de HIV-1 epidemie.

In **Hoofdstuk 8** wordt de genetische stabiliteit bestudeerd van een potentiële HIV-1 vaccin stam met grote deleties in de genen voor Vpr en Nef en in het U3 gedeelte van de LTR promoter. Dit virus heeft een sterk gereduceerd replicatievermogen in zowel getransformeerde als primaire cellen, en de homologe SIV variant is met succes gebruikt als een verzwakt, levend virus vaccin. Ook van dit mutant virus verbeterde het replicatievermogen in de loop van een geprolongeerd infectie experiment van enkele maanden. Het revertant virus vertoont een duplicatie van drie Sp1 bindingsplaatsen in de LTR, wat een promoter oplevert met zes opeenvolgende Sp1 elementen. Deze variant promoter is meer actief in transiente transfectie experimenten dan de mutant promoter waarmee het infectie-experiment was begonnen, en verbetert de replicatie van het mutant virus op een dramatische manier. Hieruit wordt afgeleid dat verzwakte stammen van HIV-1 genetisch instabiel kunnen zijn en mogelijk kunnen evolueren naar beter replicerende en wellicht meer virulente vormen. Het gebruik van zulke levend verzwakte varianten van HIV-1 als virus vaccin dient daarom opnieuw te worden bekeken.

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Koen

# Curriculum Vitae

Koen Verhoef werd geboren op 14 augustus 1968 te Huizen. Na het behalen van het V.W.O. diploma aan het Stad en Lande college te Huizen begon hij in 1986 met de studie Biologie aan de Vrije Universiteit te Amsterdam. Het doctoraal diploma Biologie werd in 1993 behaald, waarna hij enige tijd werkzaam was als onderzoeker bij enzym-producent MRC Holland. In 1994 begon hij zijn promotieonderzoek als assistent in opleiding bij dr. B. Berkhout, afdeling Humane Retrovirologie, Academisch Medisch Centrum, Universiteit van Amsterdam. Het onderzoek richtte zich op de regulatie van transcriptie van het Humaan Immunodeficiëntie Virus type 1 (HIV-1), met name op de structuur en functie van het virale Tat transactivatie eiwit. De resultaten van het onderzoek staan beschreven in dit proefschrift. Eind 1998 - begin 1999 heeft hij als EMBO fellow gewerkt bij prof. dr. H. Bujard, Zentrum für Molekulare Biologie, Universiteit van Heidelberg. Na zijn promotie zal hij op de afdeling Humane Retrovirologie werkzaam zijn op het gebied van HIV-1 drug-resistentie en vaccin-ontwikkeling.