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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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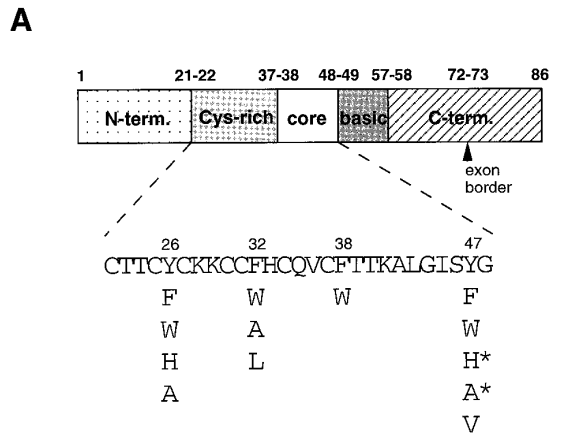


B

	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 α-helical conformation for the stretch of basic residues constituting the nuclear localization/RNA-binding domain (Mujeeb *et al.,* 1994) and an amphipatic α-helix for the core region (Loret *et al.,* 1991). In a separate study with the Tat 1–37 domain fused to the α-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 μg/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 μg pcDNA3–Tat and 1 μg LTR–CAT (COS cells), 1 μg of pcDNA3–Tat and 1 μg LTR–CAT (HeLa), or 2 μg of pcDNA3–Tat (HLCD4-CAT). Five million SupT1 T cells were transfected transiently by electroporation with 1 μg of pcDNA3–Tat and 2.5 μg of LTR–CAT reporter construct (Koken *et al.,* 1994a). SupT1 T cells and PBMC were electroporated with 1 and 2 μg, respectively, of the HIV-1 molecular clone pLAI, and 0.5×10^6 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 μl 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 μg 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-

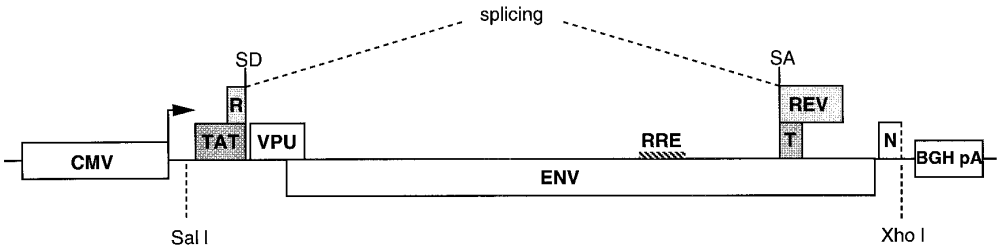


FIG. 2. Schematic of the Tat expression vector pcDNA3–Tat. Shown are the vector-derived Cytomegalovirus (CMV) immediate-early promoter (transcription start site is marked by an arrow) and the polyadenylation signal of the bovine growth hormone gene (BGHpA). *Sal*1 and *Xho*1 restriction sites denote 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

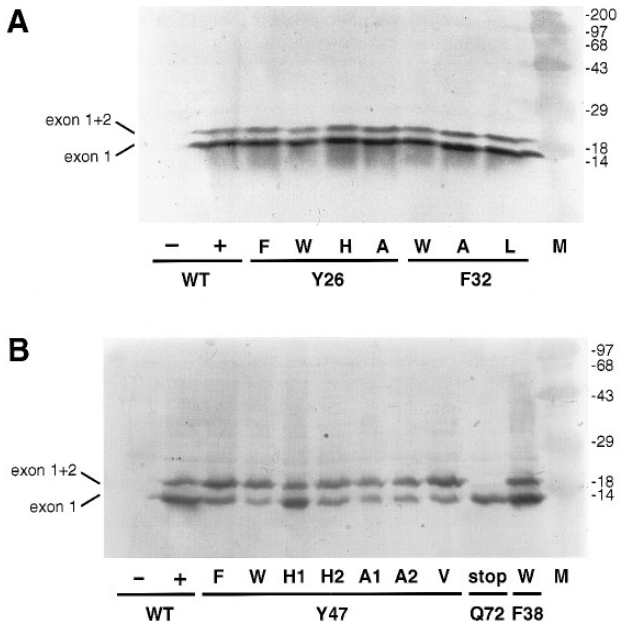


FIG. 3. Wild-type and mutant Tat proteins are stably expressed in COS cells. Western blot analysis of Tat protein expression in transiently transfected COS cells. The wild-type Tat (WT) is shown next to a mock-transfected sample (–). Lane M contains marker proteins (molecular mass in kilodaltons). The 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).

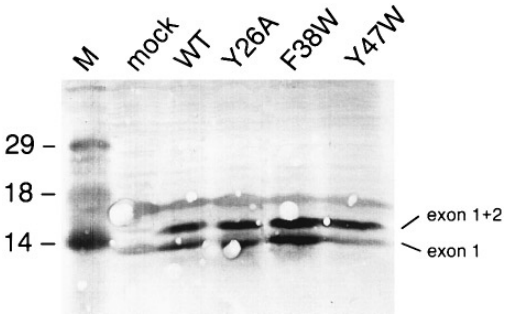


FIG. 4. Five million SupT1 T cells were electroporated with 50 μg of the individual Tat plasmids, and harvested on Day 2 post-transfection. The immunoblot was stained with mAb 4. See Fig. 3 for further details.

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).

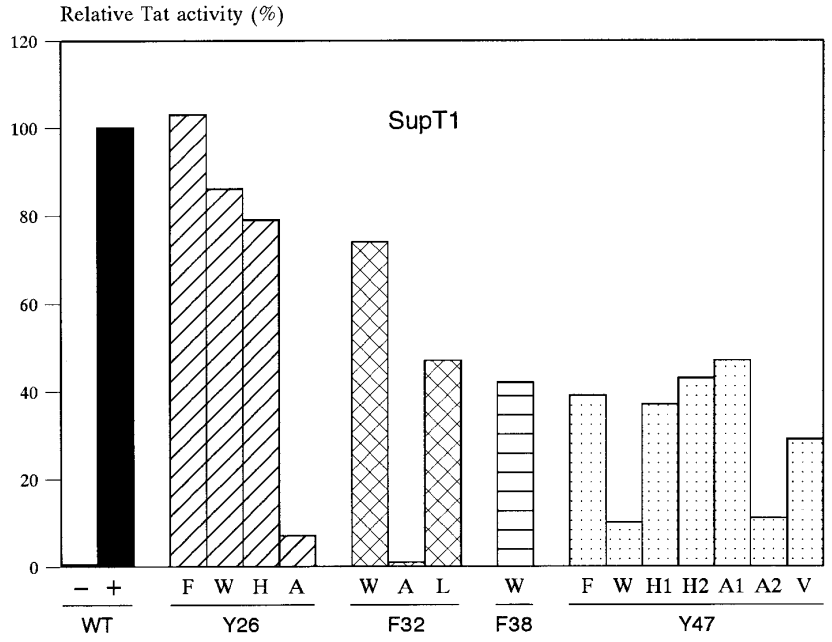
We next tested the complete set of Tat mutants in viral replication studies to determine the level of Tat activity required for virus replication. Furthermore, any discrepancies between the two assay systems may be indicative of nontranscriptional roles of Tat in the replication cycle. The SupT1 T cell 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%) $\gg$ F32W (74%), F32L (47%) $>$ Y47H1 (37%) $>$ F38W (42%) $\gg$ 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 ($P < 0.0005$).

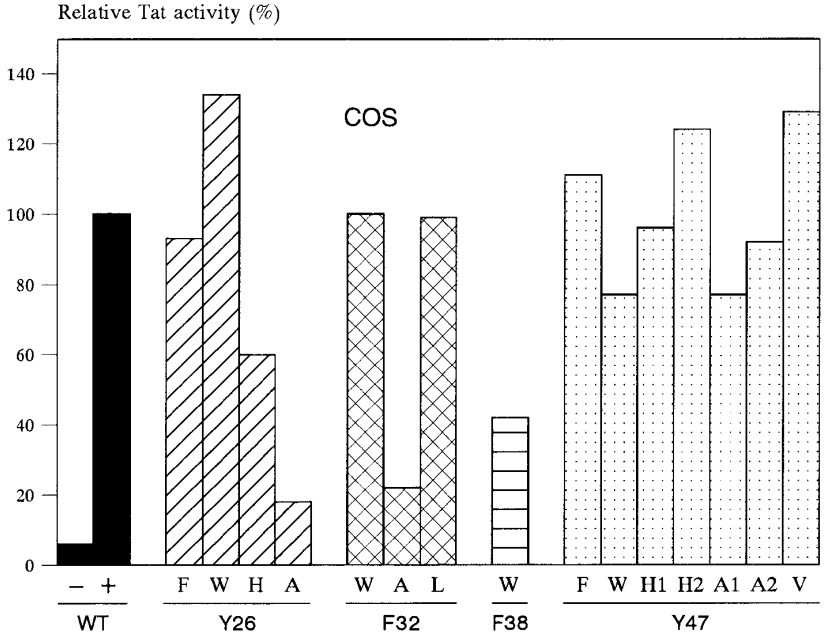
Several studies have reported considerable replication of Tat-mutated viruses in a variety of cell types (see Discussion). We therefore tested whether the replication-impaired mutants Y26A and F38W could be rescued by cellular activation with phorbol myristate acetate (PMA) and/or PHA, but no improved 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
– +
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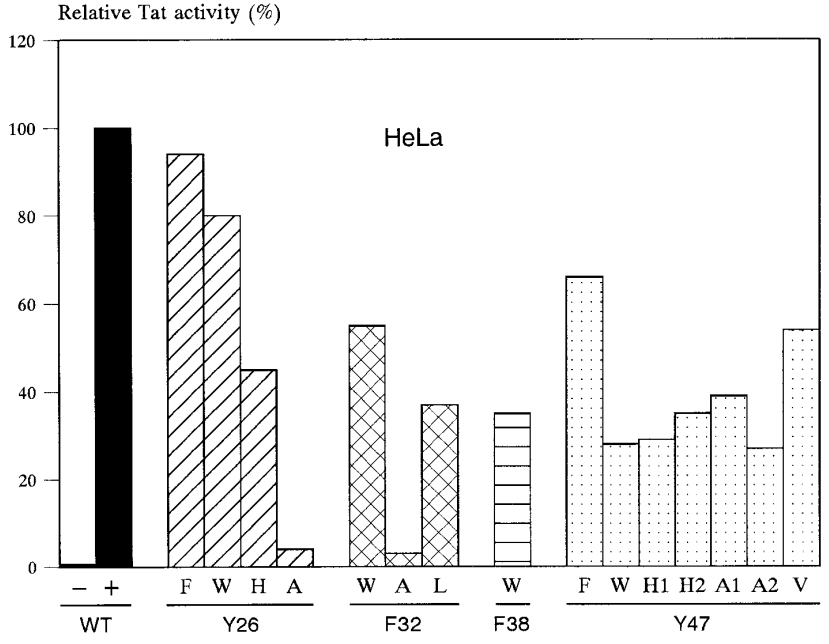
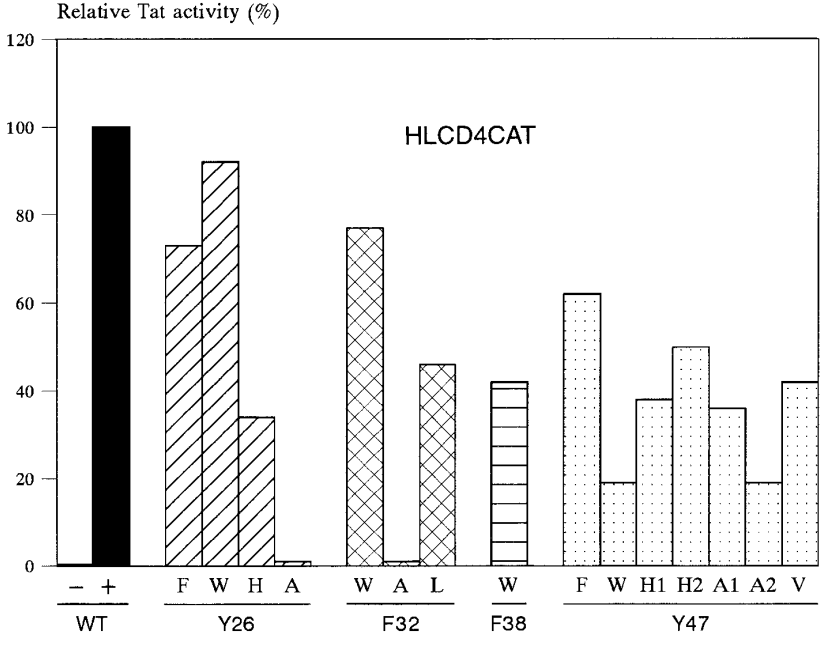
W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47


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
– +
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W A L
W
F W H1 H2 A1 A2 V
WT
Y26
F32
F38
Y47

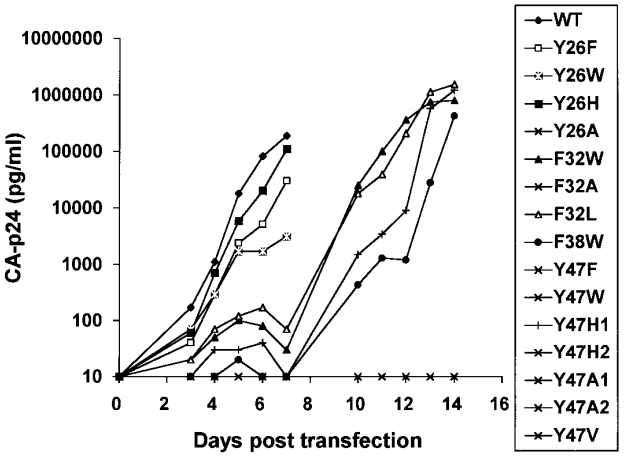

FIG. 6. Replication kinetics of Tat-mutated HIV-1 viruses. The SupT1 T-cell line was transfected with 1 μg of the wild-type and mutant constructs, and virus production was measured in the culture supernatant by CA-p24 ELISA several days post-transfection. The culture was split on Days 5 and 7 post-transfection, 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).

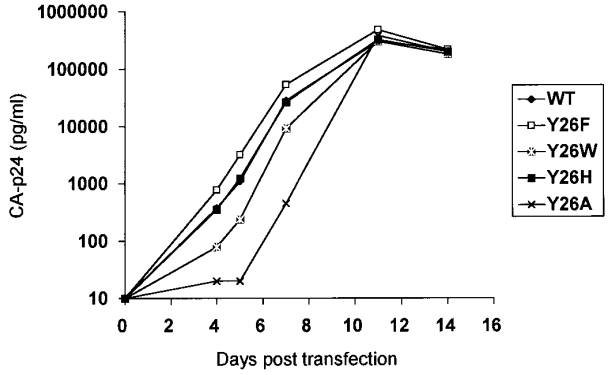

FIG. 7. Replication kinetics of Y26-mutated HIV-1 variants in primary cells. PBMCs were transfected with 2 μg of the wild-type and mutant constructs and virus production was measured in the culture supernatant by CA-p24 ELISA several days post-transfection.

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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