Recombinant Human Immunodeficiency Virus Type 1 Genomes with *tat* Unconstrained by Overlapping Reading Frames Reveal Residues in Tat Important for Replication in Tissue Culture

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Human immunodeficiency virus type 1 (HIV-1) Tat is essential for virus replication and is a potent *trans* activator of viral gene expression. Evidence suggests that Tat also influences virus infectivity and cytopathicity. Extensive structure-function studies of Tat in subgenomic settings with point mutagenesis and transient transfection readouts have been performed. These reporter assays have defined certain amino acid residues as being important for *trans* activation of reporter plasmids. However, they have not directly addressed functions related to virus replication. Here, we have studied Tat structure-function in the setting of replicating viruses. We characterized mutations that emerged in Tat during HIV-1 infections of T lymphocytes. To ensure that the selection pressure for change was directed toward protein function, we constructed HIV-1s in which the Tat reading frame was freed from constraints exerted by overlapping with the reading frames of *vpr*, *rev*, and *env*. When these recombinant viruses were passaged in T cells, 26 novel nucleotide changes in *tat* were observed from sequencing of 220 independently isolated clones. Recloning of these changes into a pNL4-3 molecular background allowed for the characterization of residues in Tat important for virus replication. Interestingly, many of the changes that affected replication when they were assayed in transient *trans* activation of plasmid reporters were found to be relatively neutral. We conclude that the structure-function of Tat in virus replication is incompletely reflected by activity measurements based only on subgenomic transient transfections.

The human immunodeficiency virus (HIV) is the etiological agent for AIDS, a disease that is characterized by a brief primary infection that is followed by a long asymptomatic period leading to disease and death. Although much has been described regarding AIDS, the detailed mechanisms underlying the initiation and development of disease remain incompletely understood. Pathogenesis of this disease is complex and likely involves both viral and host determinants (17, 33, 40, 47, 49, 52, 63, 66, 70).

One characteristic of primate lentivirus infections is the propensity for the virus to undergo rapidly genomic variations. Consequently, viruses isolated from the same patient with AIDS, at different stages of disease, often show sequence changes that phenotypically reflect differences in replication and cytopathicity. Indeed, it has been observed that HIV type 1 (HIV-1) samples recovered from patients with advanced disease tend to replicate faster and with wider cytopathicity and cellular host range than those of viruses isolated from patients in the initial stages of disease (2, 9, 14, 23, 63). Thus, one critical step in understanding AIDS and in developing strategies for intervention is a better characterization of the viral determinant(s) that contributes to virulence and/or infectivity.

Studies of the role of HIV accessory genes have implicated the integrity of *vif*, *nef*, *vpr*, and *vpu* as important for optimal viral infections in tissue culture (3, 5, 11, 13, 18, 25, 48, 57, 68). One of these accessory genes, *nef*, has also been especially well documented in monkey models as contributory toward disease progression and maintenance of high viral loads (16, 38). The statuses of *nef* and other accessory genes in a few patients have been monitored sporadically, and those limited findings are

consistent with roles for these genes in pathogenesis (17, 40, 47).

A correlation between virus load and disease has been established (12, 15, 20, 29, 30, 50, 51, 56, 65). On the basis of this principle, it is not unreasonable to consider that in addition to the accessory genes, the essential regulatory genes of HIV can contribute to virus load and thus influence virulence. In the case of Tat, there is emerging tissue culture evidence that this function affects the virus life cycle in complex ways that are not fully explainable by its *trans* activation properties on the long terminal repeat (LTR) (10, 28, 32, 44, 53). Part of this complexity is likely due to the abilities of Tat to modulate the expression of cellular genes such as major histocompatibility class I (31), tumor growth factor beta (46), interleukin 2 (IL-2) (67), IL-6 (55), tumor necrosis factor alpha and beta (6, 7, 54), manganese superoxide dismutase (24), and extracellular matrix proteins (62). Furthermore, Tat affects diverse aspects of cellular metabolism in functioning as a secreted growth factor for Kaposi-like cells (4, 21, 22). Additionally, Tat has been shown to inhibit the proliferation of antigen-specific T lymphocytes (60, 64) and to induce apoptosis in cells (45).

Many Tat structure-function studies are based on subgenomic transfection experiments. Findings from these approaches have demonstrated that the second coding exon of Tat is wholly dispensable for transcription and have defined certain amino acid residues in the first coding exon as being critical for activity (for reviews, see references 35 and 36). To date, there has not been a focused attempt to correlate subgenomic findings on plasmid *trans* activation with roles during virus replication. Hence, it is unclear how accurately and completely transient transfections of plasmid reporters measure functions related to viral infections of cells.

We wished to examine Tat structure-function in a genomic, replicating setting. In assessing the biological role(s) of TAR

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RNA (28, 41) or LTR DNA sequence (42), others have similarly evolved from reporter assays to assays of virus growth in T cells. To this end, we devised a protocol that characterizes changes in Tat during HIV-1 infections of cells. In this approach, we freed *tat* from constraints exerted by the need to maintain open, overlapping coding frames (i.e., *vpr*, *rev*, and *env*). Thus, during passages of viruses in cells, changes that emerge would more likely be due to selection for protein function. In assessing the changes that thus appeared in *tat*, we found 22 amino acid alterations (from 26 nucleotide mutations). Many of the changes agree with previous results from subgenomic studies; however, some reveal a discordance between residues important for virus replication and those important for transient *trans* activation of plasmids. Thus, there is not a strict one-for-one relationship between amino acids needed for *trans* activation of plasmids and those needed for infections.

MATERIALS AND METHODS

Plasmid constructions. All proviruses were derived from the parental pNL4-3 molecular clone (1). The Tat(-) provirus and the insertion of cDNAs into *nef* have been described elsewhere (37). Briefly, Tat(-) provirus was constructed by engineering two stop codons at amino acids 11 and 12 into the first coding exon of *tat*. cDNAs for the full-length *tat* from HIV SF2, the first coding exon of *tat*, or *tat* mutants were placed into *nef* of this Tat(-) genome in frame for expression. To characterize mutations that arise during virus replication, *tat* cDNAs were PCR amplified from infected-cell DNAs, sequenced, and cloned into the pSG5 vector (27) for functional assays. pM1ex and pNL101 were derived from the molecular clone pNL4-3. pM1ex expresses only the first coding exon of Tat in its natural location in the genome. This molecular clone was constructed by introducing a stop codon at amino acid 72. pNL101 was constructed by removing a stop codon present in the second coding exon of Tat in pNL4-3 at amino acid 86. A single nucleotide change at this location extends the second exon from 86 amino acids to the 101 form that is found in most wild isolates of HIV-1 (for a review, see reference 34).

Cells, transfection, and viral infections. The T-cell lines C8166-45 and MT2 and the Jurkat cell line were cultured in RPMI 1640 with 10% fetal calf serum. HeLa cells were propagated in Dulbecco's modified Eagle's medium (with 10% fetal calf serum). HeLa cells were transfected with calcium phosphate. T cells were electroporated with the different proviral molecular clones by using 300 V and 960 μ F (Bio-Rad). For infections, fresh T cells were inoculated with viruses normalized by the amount of reverse transcriptase. Transfected and infected cultures were kept for 30 days with periodic additions of fresh T cells; virus replication was monitored by measuring supernatant reverse transcriptase activity (69). Cell DNAs were extracted at the indicated times after infection.

Jurkat cells were infected with viruses normalized for infectivity with the CD4-LTR/ β -galactosidase indicator cell line (MAGI assay), which was obtained through the AIDS Research and Reference Reagent program (39).

Human peripheral blood monocytes (PBMC) were stimulated with phytohemagglutinin (final concentration, 250 ng/ml) for 3 days prior to infection and were maintained in RPMI 1640 with 20% fetal bovine calf serum and 10% IL-2 (Pharmacia). Usually, 10^6 PBMC in a 0.5-ml volume were absorbed with virus supernatants containing 5×10^6 cpm of reverse transcriptase (RT) activity at 37°C for 1 h.

DNA analysis. The first coding exon of *tat* or the full-length *tat* cDNA from chimeric proviruses was PCR amplified from infected-cell DNA with an *env-nef* primer pair (sense [5'-CGC TGA ATTY CAG ACA GGG CTT GGA AAG G-3'] and antisense [5'-ATT GGG ATC CAT TGC TAC TTG TGA TTG-3']). The double-stranded DNA products were digested with *Bam*HI and *Eco*RI and were cloned into pUC19. Sequencing was performed by the dideoxy-chain termination method.

Southern blotting analysis was performed by transferring the PCR-amplified fragments from a 1.6% agarose gel to a nylon membrane (Amersham). The membrane was hybridized with a 32 P-radiolabeled *tat* probe.

CAT reporter assays. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (26). After resolution by thin-layer chromatography, the radioactive spots were quantitated with a Fuji PhosphorImager. For quantitations, CAT activities were analyzed in the linear range of acetylation. All transfections and corresponding assays were done three to five times.

RESULTS

Construction of HIV-1 proviruses with *tat* unconstrained by overlapping reading frames. Many investigators have mutagenized extensively the first coding exon of Tat. Indeed, Tat function has been well-studied in subgenomic transient re-

FIG. 1. Construction of Tat(-) proviruses that express the first coding exon of *tat* or both coding exons of *tat*. Schematic representations of the pNL4-3 virus (1) and the Tat(-) proviruses are shown. Tat(-) genomes possess the same backbone as pNL4-3, but the *tat* gene was inactivated by engineering two consecutive stop codons at amino acids (aa) 11 and 12. pTat(-)2ex and Tat(-)1ex are derivatives of Tat(-) provirus, with either the first coding exon of *tat* or full-length (two-coding-exon) *tat* inserted in the correct translational frame in *nef*.

porter assays (for reviews, see references 34 and 36). Dissection of Tat function(s) in replicative settings has been minimal. Thus, although we know much about the properties of Tat in activating LTR reporters, how these results correlate with the effects of Tat on virus growth are poorly known (10, 32, 44, 53).

We wished to analyze Tat structure-function in virus replication assays. We based our analysis on visualizing the emergence of mutations in *tat* during virus passages in T cells. To minimize extraneous constraints that might hinder selection based on function, we separated *tat* from the normal organizational requirement used by the virus to keep open four overlapping reading frames (i.e., *tat*, *vpr*, *rev*, and *env*). This was done by first constructing a Tat(-) provirus in which *tat* was inactivated by two stop codons placed at amino acids 11 and 12 (Fig. 1). We complemented *in cis* for function by inserting cDNAs for either the two coding exons [Tat(-)2ex] or the first coding exon [Tat(-)1ex] of *tat* from HIV-1 SF2 (Fig. 1) into the *nef* reading frame of this Tat(-) provirus.

The second coding exon of *tat* contributes to viral replication. We electroporated T-cell C8166-45 with Tat(-)2ex or Tat(-)1ex molecular genomes. Viruses produced from this initial electroporation were normalized for RT activity and were used to infect fresh cells. These infected cells were monitored for virus growth by measuring RT production every 2 or 3 days over a period of approximately 30 days. In this context, we observed a distinct delay (approximately 6 days) in virus replication for Tat(-)1ex virus compared with Tat(-)2ex virus (Fig. 2A). The transfection-infection experiments were repeated six times, and this delay was observed each time. When we reproduced the infection in PBMC, an even larger difference in virus growth profiles was found (Fig. 2B). In this cellular background, the one-exon virus replicated very poorly.

A)

B)

FIG. 2. Tat(-)1ex shows a delay in virus replication compared with Tat(-)2ex. Typical viral growth curves defined by supernatant RT (in counts per minute per microliter) assays are shown. (A) C8166-45 T cells were electroporated with the molecular clone Tat(-)2ex or Tat(-)1ex. Fresh C8166-45 cells were infected with the viruses produced from electroporations. Growth curves for Tat(-)2ex and Tat(-)1ex are shown from RT samples collected every 3 days. (B) PBMC (10^6) infected with normalized supernatants derived from transient transfections of molecular clones Tat(-)1ex or Tat(-)2ex into HeLa cells. For each infection, 5×10^6 cpm of RT equivalent of virus was used.

Thus, unlike subgenomic transient findings with plasmid reporters in which the second exon of Tat was negligibly important for transcription (for reviews, see references 34 and 36), these virus infections strongly suggest that the second exon of *tat* has a significant role in HIV-1 replication, especially in primary cells.

Characterization of *tat* mutants generated during viral replication. We wondered whether unconstrained *tat* might selec-

tively evolve changes during passaging. Using specific primer pairs (see Materials and Methods), we amplified *tat* sequences from proviruses in infected cells. These sequences were ligated into plasmids, and 140 *tat*-containing clones were isolated from cells infected with the Tat(-)1ex virus. A total of 80 additional clones were similarly isolated from infections with the Tat(-)2ex virus. We found that when normalized for equivalent numbers of infected cells, the *tat* amplification signal from

FIG. 3. PCR amplification of *tat* sequences from infected cellular DNA. (A) At 30 days after infection, cellular DNA was extracted from mock-infected cells (lane 1), cells infected with pNL4-3 (lane 2), cells infected with Tat(–)1ex (lane 3), or cells infected with Tat(–)2ex (lane 4). PCR amplification with the *env-nef* primer pair generated an amplified fragment of 171 bp for pNL4-3, a fragment of 370 bp for the provirus containing only the first coding exon of *tat*, and a fragment of 394 bp for the provirus containing the two coding exons of *tat*. (B) To verify that the amplified fragments were *tat* specific, the PCR products were transferred to a nylon membrane and hybridized with a ³²P-labeled *tat* probe. Lane 1, mock-infected cells; lane 2, cells infected with pNL4-3; lane 3, cells infected with Tat(–)1ex; lane 4, cells infected with Tat(–)2ex.

cells infected with Tat(–)1ex (Fig. 3, lane 3) was consistently weaker than that from cells infected with Tat(–)2ex (Fig. 3, lane 4); this is consistent with the above observation that the latter virus replicates more efficiently (Fig. 2).

We sequenced all 220 clones completely. A total of 26 nucleotide mutations (data not shown) that specified for 22 discrete amino acid changes were found (Fig. 4). Each mutant was found once during this screening. This suggests that no one change conferred a dominant selective advantage to the virus, which would have resulted in the outgrowth of a major *tat*-containing population. However, we cannot formally exclude that some mutations might be advantageous for the virus but are balanced by countervailing changes elsewhere in the genome. Below (see Fig. 6), we examined this issue by transferring mutant Tat cDNAs into isogenic viral genomes for side-by-side comparisons.

trans activation activity of *tat* mutants. To characterize more fully the changes in Tat, we first tested each mutant for activity in subgenomic plasmid transfections. After being cloned into eukaryotic expression vector pSG5 (27), Tat-containing plasmid was cotransfected into HeLa cells with an HIV-1 LTR-CAT reporter. At 48 h after transfection, CAT activities were measured. See Fig. 6 for the range (from 50 to 190% of the wild type) of *trans* activation activities exhibited by the respec-

A)

B)

FIG. 4. Analysis of *tat* mutants produced after infection of T cells with the Tat(–)2ex virus or the Tat(–)1ex virus. Amino acid changes found in Tat(–)2ex (A) and Tat(–)1ex mutants are shown. wt, wild type.

FIG. 5. Tat mutants show similar activities in transient plasmid transfections. Mutants isolated from tissue culture selection were subcloned into expression vector pSG5 (26) and were tested for their capacity to *trans* activate an HIV-1 LTR-CAT plasmid. (A) HIV-1 LTR CAT was transfected alone into HeLa cells or cotransfected with wild-type two-exon Tat cloned into pSG5 or with different two-exon Tat mutants cloned into pSG5. CAT activities were assayed 48 h after transfection. The activities of the different Tat two-exon mutants were calculated relative to the wild-type two-exon Tat activity, which was set at 100%. The values reported are the average of three separate experiments. (B) The same protocol was used to determine the CAT activities of the one-exon Tat mutants. In this case, activity was plotted relative to that of the wild-type one-exon Tat.

tive mutants. For example, pMD61N, which is a one-coding-exon version of *tat* with a D-to-N mutation at amino acid 61, kept only 47% of the wild-type activity. Mutant pME9G, with an E-to-G mutation at amino acid 9, had activity that was 190% that of the wild type (Fig. 5B). Similarly other mutants were either slightly higher (e.g., pM44D and pA58T) or slightly lower (e.g., pNL43F or pMF38LR40K) in activity (Fig. 5A).

Growth kinetics of chimeric viruses containing Tat mutants.

The function of Tat in virus replication is complex (32). To examine how mutations might affect growth of virus in T cells, we transferred the mG44D, mF38LR40K, mD5N, mK90R, mL43F, mP81FS, and mR55Q mutants individually into the *nef* locus of an HIV-1 Tat(-) molecular clone. These mutants are representative of changes that slightly increased, slightly decreased, or did not change activity in transient transfections (Fig. 5). The resulting seven chimeric viral genomes were tested for replication in T cells. We found that Tat(-)mD5N virus (with a mutation in the acidic N-terminal domain) and one other virus with a mutation in the basic domain (i.e., mR55Q) replicated, but with delayed kinetics (Fig. 6, top and bottom). Three other viruses with mutations in the core do-

main (i.e., mL43F, mF38LR40K, and mG44D) failed to replicate (Fig. 6, top and center). A sixth virus, Tat(-)mP81FS, containing a frameshift in the second coding exon of *tat* was delayed in virus production (Fig. 6, center), while a seventh virus, Tat(-)mK90R, which has a conservative change in the second coding exon of *tat* (K90R), grew with kinetics similar to those of a wild-type two-exon Tat virus (Fig. 6, top).

It was unexpected that when the Tat mutants were assayed by transient *trans* activation with the pSG5 vector they showed relatively minor differences in activities (Fig. 5) yet when the same set of mutants was assayed by virus replication (Fig. 6), major discrepancies were seen. This suggests that mutation findings from transient plasmid transfections may not relate directly (neither quantitatively nor qualitatively) to effects visualized in virus infections. To verify this and to rule out that the observed differences between replication and plasmid *trans* activation did not arise artifactually as a consequence of altered levels of Tat when it was expressed from proviral genomes (as opposed to when it was expressed from pSG5), we performed two additional sets of plasmid transfections. In the first, the Tat(-)mG44D, Tat(-)mF38LR40K, Tat(-)mD5N, Tat(-)mK90R, Tat(-)mL43F, Tat(-)mP81FS, and Tat(-)mR55Q proviral genomes were cotransfected separately into HeLa cells with an LTR-CAT plasmid. CAT activities were measured 48 h later (Fig. 7A). Similarly to the findings from pSG5 (Fig. 5), when each of the Tat mutants was expressed from a complete viral genome, it produced comparable *trans* activation of cotransfected LTR-CAT plasmid (Fig. 7A). As seen in the pSG5 experiments (Fig. 5), there was a range of activities, but in no case were the differences so remarkable that they could have predicted that some mutants were to be wholly incapable of supporting virus replication. In a second series, each proviral plasmid was individually transfected into HeLa cells, and the resulting production of supernatant reverse RT was measured (Fig. 7B). We compared side-by-side production of RT from wild-type two-exon molecular plasmid and two-exon Tat mutant molecular plasmids (Fig. 7B, top) and wild-type one-exon and one-exon Tat mutant molecular plasmids (Fig. 7B, bottom). Because RT production after plasmid transfection is a reflection of transient *trans* activation by Tat of the respective *cis* LTRs, the relative similarities between the RTs within the two sets are consistent with the suggestion that there is no fundamental difference in relative protein levels when Tat is expressed from a proviral format as opposed to a pSG5 format.

Role in virus replication of the second coding exon of *tat* when it is expressed from its natural location in the HIV-1 genome. Our results suggested a role for the second coding exon of *tat* during viral replication. This was shown by using a construction in which the *tat* gene transferred into the *nef* reading frame (Fig. 6). To verify this result in the setting in which *tat* is expressed from its normal location in the viral genome, we constructed two new HIV-1 molecular clones that synthesize Tat of either 72 amino acids (pM1ex) or 101 amino acids (pNL101) (Fig. 8A). pM1ex was constructed by introducing a stop codon by changing a C to a T at amino acid 72. Since most nonlaboratory strains of HIV-1 Tat protein contain 101 amino acids (for a review, see reference 34), we released the stop codon found in pNL4-3 after amino acid 86 by a single nucleotide change which extended the open reading frame to 101 amino acids. Thus, pNL101 is a pNL4-3 virus that has a 101 (instead of 86)-amino-acid Tat protein. We noted that neither the nucleotide change in pM1ex nor that in pNL101 disturbed the open reading frames of overlapping genes (i.e., first coding exon of *rev* for pM1ex and second coding exon of *rev* and *env* for pNL101) (Fig. 8A, bottom).

FIG. 6. Replication kinetics of proviruses containing mutated *tat* genes. Tat(-) molecular clone was used to construct chimeric viruses containing different Tat mutants. Molecular clones containing mD5N, mG44D, mK90R, mL43F, mR55Q, mP81FS, or mF38LR40K, respectively, were individually transfected into HeLa cells. At 48 h later, the viruses produced were normalized and used to infect fresh T cells (C8166-45). Equivalent inoculum of each virus was used to infect T cells (RT, 0.5×10^6 cpm/ 10^6 cells). The graphs are separate experiments representative of six (top), four (middle), and two (bottom) replications.

FIG. 7. When expressed from a proviral genome, different Tat mutants are expressed to similar levels. (A) An HIV-1 LTR-CAT plasmid was transfected alone (lanes 1, 8, and 12) or was cotransfected with Tat(-)2ex molecular clones (lanes 2, 9, and 13), Tat(-)1ex molecular clones (lanes 3 and 10), or chimeric virus genomes containing mutant mD5N (lane 4), mK90R (lane 5), mL43F (lane 6), mR55Q (lane 7), mG44D (lane 11), mP81FS (lane 14), or mF38LR40K (lane 15). (B) Representation of RT activities from HeLa cells transfected individually with molecular clones containing the different Tat mutants. Top graph, side-by-side comparisons of wild-type Tat(-)2ex with two-exon Tat mutant proviruses; bottom graph, a side-by-side comparison of wild-type Tat(-)1ex with a single one-exon Tat mutant provirus. RT values were measured as described elsewhere (68).

Virus stocks of pM1ex and pNL101 were obtained after transfection into HeLa cells. Two separate cell lines were used to compare the replication kinetics of the two viruses. We found a distinct delay in growth of the pM1ex virus compared with that of the pNL101 virus in both MT2 and Jurkat cells (Fig. 8B). In the former, the delay in peak RT was 9 days; in the latter the delay was 4 days (Fig. 8B). On the basis of the finding of Dimitrov et al. (19), this suggests that the replication of pM1ex is 1 to 3 log units worse than that of pNL101. These results for viruses that produce the second coding exon of Tat from its normal locale confirm the findings for viruses that express Tat positioned into *nef* (Fig. 3 and 6).

DISCUSSION

Understanding the determinants of virus replication during HIV-1 infections is important for developing targeted interventions. Tat is an essential protein for virus viability that has a role(s) in replication. Tat has also been shown to have pleiotropic effects on cellular genes and cellular metabolism (4, 6, 7, 21, 22, 24, 31, 45, 46, 54, 60, 62, 64, 67). Presumably, an integration of Tat's effects on the viral LTR and on host cellular genes reflects its total role(s) during productive HIV infections.

Because available data on Tat structure-function are from subgenomic studies and because subgenomic transfections account for only one part of the activities important for virus replication, we developed an approach to monitor Tat function in infections. We reasoned that from the emergence of mutations in Tat, one should gain insights about important protein residues. If a mutation appears, two deductions could be advanced. (i) The particular change is neutral for replication. In this case, one would find occasional occurrence of this mutation, since the change does not confer a growth advantage. (ii) The mutation provides a gained advantage for replication. Here, in principle, viruses carrying this change should outgrow other viruses, unless the gained advantage in *tat* is balanced by a second disadvantage in another part of the genome. In our experiments, we controlled for this latter possibility by transferring each mutation into otherwise isogenic molecular backgrounds when comparing effects on virus growth (Fig. 6). There is a third possible deduction. This comes from observations that some regions of Tat are privileged in never exhibiting changes. Such regions are presumably critical for replication. Changes occur but are not propagated because they are nonviable for the virus.

In our virus replication assays, 22 nucleotide changes (data not shown) emerged in the first coding exon of *tat*. A total of 27.3% of these mutations were located in the N-terminal domain, some disturbing its acidic composition (e.g., mutant mD5N). Interestingly, changes such as mD5N have been shown previously by others (and have been confirmed by us [Fig. 5B]) in transient transfections not to affect Tat *trans* activation (for reviews, see references 34 and 36). Two mutations in nucleic acid sequence were found in the cysteine-rich domain. However, these were conservative for amino acids (mN23NR55Q and mK28KS75S), supporting prior findings (for reviews, see references 34 and 36) that the cysteine-rich region is poorly tolerant of changes.

Another 27.3% of the mutations were in the core region. We found a nucleotide mutation at K41, a position previously shown to be functionally critical (62); however, the nucleotide change conserved the amino acid sequence (mK41K). We did find one missense mutation in the core domain (mF38L). The phenotype of change at Phe-38 has been variously argued to be deleterious in some subgenomic assays (59) and nondeletori-

A)

	68				72	
	S	L	S	K	Q	Tat
WT	TCT	CTA	TCA	AAG	CAG	
		L	Y	Q	S	Rev
					stop	Tat
pM1ex				AG	TAG	
C→T				S		Rev

	84				86	
	P	K	E	stop		Tat
WT	CCG	AAG	GAA	TAG	AAG	
	R	R	N	R		Rev
		E	G	I	E	Env
		E	S	K		Tat
pNL101		GAA	TCG	AAG		
A→C		N	R			Rev
		I	E			Env

B)

FIG. 8. Growth comparisons of HIV-1 with one-exon Tat expressed from the authentic genomic locus (pM1ex) with an otherwise isogenic virus expressing a 101-amino-acid two-exon Tat protein (pNL101). (A) Schematic representations of the pM1ex and pNL101 molecular genomes. pM1ex and pNL101 possess the same molecular backbone, except that their respective Tat proteins encode a 72- or a 101-amino-acid (aa) polypeptide. In pM1ex, this was done by substituting a stop codon at amino acid 72, and in pNL101 this was done by removing the stop codon at amino acid position 87, correcting the reading frame to 101 amino acids. Sanger dideoxy sequencing shows the C-to-T point mutation that introduces the stop codon at amino acid 72 and the A-to-C change that releases the stop codon after amino acid 86 (bottom). Neither change affects the Rev or Env open reading frames. WT, wild type. (B) Molecular clones pM1ex and pNL101 were individually transfected into HeLa cells. At 48 h later, viruses were normalized for RT (RT, 0.5×10^6 cpm/ 10^6 cells) and were used to infect MT2 cells or were normalized for infectivity (equivalent of 50 infectious units per 10^6 cells) by using the MAGI assay and were used to infect Jurkat cells. Virus growth in both cell lines was monitored. The one-exon virus was found to be consistently delayed for peak RT production by 4 days in Jurkat cells and by 9 days in MT2 cells compared with the two-exon virus. Each set of experiments was done twice.

ous in others (for a review, see reference 34). In our assay, this Tat mutant transiently *trans* activated the LTR-CAT reporter well (Fig. 5). However, a virus with this mutation [Tat(-)mF38LR40K] was growth defective (Fig. 6). This represents another example in which transient transfection results do not necessarily reflect the biology of virus replication. We interpret Phe-38 changes as down mutations that are compatible with reduced virus growth, permitting changes at this position to be seen in initial screenings.

A total of 36.4% of the mutations were found in the basic domain. In subgenomic settings, this domain has been shown to be highly permissive for change (for reviews, see references 34 and 36). The basic domain has a sequence of nine amino

acid residues, RKKRRQRRR (residues 49 to 57), which subserves the RNA-binding function of Tat. Although a single arginine residue at position 53 flanked on each side by three or four basic amino acid has been proposed to be sufficient for binding to TAR RNA (8), other basic amino acid residues and their positions relative to that of the arginine presumably provide added electrostatic contacts (8, 61). We saw four missense mutations in this stretch of amino acids. mN23NR55Q and mR55Q changed R-55 to Q; mL43LR57D changed R-57 to Q; and mR52Q changed R-52 to Q. We found that none of these mutations significantly affected transient *trans* activation of plasmids by Tat (Fig. 5 and 7). However, when one of these mutations was tested in virus (Fig. 6 [mR55Q]), it produced a

12-day replication delay compared with formatted wild-type Tat-virus.

Of the 80 clones sequenced from the Tat(-)2ex virus, we found 18 nucleic acid changes (data not shown). Surprisingly, a small fraction (4 changes) of the total was located in the second coding exon of *tat*. Furthermore, two of the four changes were conservative for amino acid (i.e., mE92E and mK28KS75S), and a third was a synonymous change (mK90R). The fourth mutation, mP81FS, was a deletion of one nucleotide that resulted in a frameshift at residue 18 creating a new C terminus, rich in basic amino acids. Interestingly, in plasmid transfection assays, mP81FS retained the same activity as the wild-type two-exon *tat* (Fig. 5). However, mP81FS virus was distinctly delayed in replication (Fig. 6). These findings are interesting in light of prior subgenomic findings that the second coding exon of *tat* is wholly dispensable for *trans* activation of plasmid reporters (35, 43, 58). Our virus assays provide some biological qualifications to those subgenomic observations. Indeed, the Tat(-)1ex virus does replicate in a *tat*-unconstrained genomic format; however, invariably, this virus is growth delayed compared with Tat(-)2ex virus, especially in primary cells (Fig. 2). This result, together with the observation that in passaging *tat*-unconstrained viruses very rarely do changes occur in the second exon of *tat*, indicates that integrity of this exon is important for virus replication. We also confirmed this importance using HIV-1s that expressed either a 72- or a 101-amino-acid Tat protein from the authentic genomic formats (Fig. 8). The growth kinetics of these two viruses in Jurkat and MT2 cells were compared (Fig. 8B). In side-by-side cultures, the virus encoding one-exon Tat was consistently delayed by 4 to 9 days compared with an otherwise isogenic virus encoding a full-length 101-amino-acid Tat protein.

Our goal was to study Tat structure-function during virus replication and, in doing so, to verify whether findings from subgenomic assays accurately reflect virus biology. From our experiments, we conclude that transient transfection assays predict partially Tat function(s) in productive infections. There are observable differences in LTR-plasmid *trans* activation and virus replication. One simple explanation, which does not exclude others, is that during virus infections Tat needs to *trans* activate cellular genes whose expression benefits HIV (6, 7, 24, 46, 54, 62, 67). Domains in Tat necessary for this activity may coincide with only a subset of that needed for activation of the LTR.

We caution that our approach in studying changes that emerge during replication is based on freeing *tat* from overlap with *vpr*, *env*, and *rev*. Thus, the frequency and the quality of the mutations found in our study might be different from what might emerge from passaging HIV-1 in its normal genomic organization. We do note that this unconstrained approach is necessary because if overlaps are not removed, most amino acid changes in Tat cannot be analyzed without incurring multiple genetic effects. In the limited instances in which we could alter Tat without affecting overlapping reading frames (Fig. 8), the results are compatible to findings that emerge from placing *tat* into *nef*. In general, we suggest that whenever possible, one should confirm function and biology in virus-specific settings.

ACKNOWLEDGMENTS

This study was funded in part by the AIDS Targeted Antiviral Program from the NIH Office of the Director.

We thank M. Benkirane, R. Chun, S. Smith, and A. Blauvelt for critical reading of the manuscript.

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