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Missense mutations in an infectious human immunodeficiency viral genome: Functional mapping of *tat* and identification of the *rev* splice acceptor

(site-directed mutagenesis/cloned human immunodeficiency virus type 1 provirus/trans-regulation/virus expression)

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ABSTRACT **Single nucleotide alterations were introduced into an infectious clone of human immunodeficiency virus type 1 to create a series of missense mutants in the *tat* coding region. Although mutations in a proline-rich region and a basic lysine-arginine-rich region resulted in wild-type phenotypes, five of six mutations in a cysteine-rich domain completely abolished *tat* activity and virus replication. One cysteine mutant retained *tat* activity but was negative for virus expression. Surprisingly, this mutant could not be complemented by *tat*, and virus expression was restored only by cotransfection with a plasmid expressing the *rev* gene. Another mutant with an alteration toward the C-terminal region showed significantly reduced *tat* activity and required complementation by a combination of *tat* and *rev* for virus replication. Further analysis revealed that a previously unrecognized splice acceptor site within this region, apparently used to generate the rev mRNA, had been altered. We provide evidence suggesting that tat and rev proteins are encoded by distinct mRNA species.**

Human immunodeficiency virus (HIV), the causative agent of acquired immune deficiency syndrome (AIDS) contains three major structural genes (*gag*, *pol*, and *env*) in common with all retroviruses (1). In addition, HIV contains several accessory genes—namely, *vif* (virion infectivity factor) (2), *vpr* (viral protein R) (3), *tat* (trans-activator) (4), *rev* (regulator of expression of virion proteins) (5), and *nef* (negative factor) (6); these designations were proposed recently to standardize the nomenclature for known human retrovirus accessory genes (7). The *tat*- and *rev*-encoded proteins have both been shown to be required for virus replication. *Tat* activates transcription from the viral long terminal repeat (LTR), resulting in increased levels of viral RNA and proteins (8). The *rev* gene product allows accumulation of steady-state levels of gag–pol and env mRNAs (5) and, in addition, has been shown to negatively affect transcription (8). Also *rev* has been proposed to function as an anti-repressor at the translational level (9). The trans-regulatory genes, *tat* and *rev*, are expressed from largely overlapping reading frames. To identify domains of these proteins that are important for function, we have generated a series of HIV-1 proviruses containing altered coding sequences in the *tat* and *rev* genes. Site-directed mutagenesis was targeted to the three major structural motifs of the functional first coding exon of the *tat* gene—the N-terminal proline-rich region, the middle cysteine-rich region, and the C-terminal basic amino acid stretch. Alterations in the last of these regions affected the first *rev* coding exon as well.

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The mutants were evaluated for phenotypic changes by transfection into COS-1 cells and assaying (*i*) their ability to trans-activate the homologous LTR by chloramphenicol acetyltransferase (CAT) assay, (*ii*) viral mRNA synthesis by Northern (RNA) blot, (*iii*) viral protein synthesis by radioimmunoprecipitation (RIP) assay, (*iv*) virus production by electron microscopy and reverse transcriptase assay, and (*v*) in the case of replication-defective mutants, their ability to be complemented by *tat*/*rev* cDNA clones.

The missense mutants thus characterized can be divided into four groups: (*i*) replication-competent mutants having the wild-type phenotype, (*ii*) replication-defective mutants complemented by a functional *tat* gene, (*iii*) replication-defective mutants complemented by *rev*, (*iv*) replication-defective mutants complemented by *tat* and *rev*. Of the six mutants with altered cysteine residues, five exhibited group *ii* and one exhibited group *iii* phenotype. Another mutation within *tat*, upstream of the *rev* coding sequence, resulted in a decrease of both *tat* and *rev* activities. We subsequently identified a distinct splice acceptor site, used by the *rev* gene, which is altered in this mutant.

MATERIALS AND METHODS

Construction of Missense Mutants. Missense mutants were constructed using a subclone of HIV containing the *tat* and *rev* coding region, which was then substituted into the parent wild-type vector pHXB2gpt (10) as described (8). Briefly, oligonucleotides (21 bases in length) containing single-base substitutions, as indicated in Fig. 1, were used in the double-primer extension procedure (12).

Stable mRNA Analysis. Twenty micrograms of total cellular RNA from COS-1 cells transfected with wild-type or missense mutants were applied to 1% agarose/formaldehyde gel and processed for Northern (RNA) analysis (13). For S1 nuclease analysis, RNA was hybridized to ^{32}P-5′ end-labeled synthetic oligonucleotide probe, complementary to the sequence shown in Fig. 5*A*. Hybridization and S1 nuclease digestion were done by standard procedures (14). Reaction products were analyzed on a 15% polyacrylamide/8 M urea gel.

Analysis of Viral Proteins. COS-1 transfectants were labeled with [^{35}S]methionine and [^{35}S]cysteine as described (8). RIP assay (15) of viral proteins was done using a pooled serum from seropositive patients at 1:10 dilution. RIP prod-

Abbreviations: HIV, human immunodeficiency virus; *vif*, virion infectivity factor; *vpr*, viral protein R; *tat*, trans-activator; *rev*, regulator of expression of virion proteins; *nef*, negative factor; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; RIP, radioimmunoprecipitation; nt, nucleotides.

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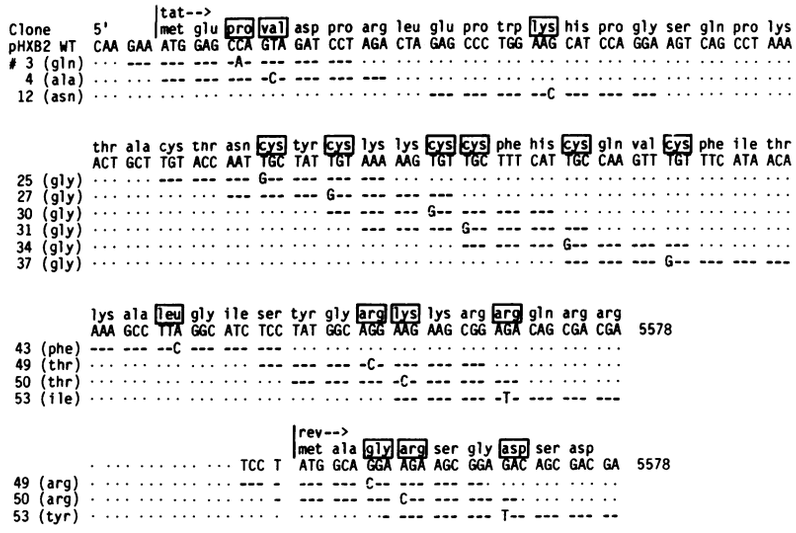

FIG. 1. The relevant sequences of the *tat* and *rev* genes of the functional proviral clone HXB2 are shown. Mutant clones numbered according to the positions of the altered amino acids contain single amino acid changes within each of the three major structural motifs of the *tat* gene. The amino acid changes are as indicated. Mutant sequences were confirmed by DNA sequencing (11).

ucts were separated on a 10% sodium dodecyl sulfate/polyacrylamide gel. Fluorography was done with an autoradiography enhancer, Enlightning, from New England Nuclear.

RESULTS

***tat* Activity of the Mutant Proviruses.** The mutant proviruses were tested in COS-1 cells for their ability to trans-activate the homologous LTR linked to the CAT indicator gene (16). All mutants with altered cysteine residues, with one exception (mutant 31), had markedly reduced ability to trans-activate. Mutant clone 43 was less compromised in its ability to trans-activate, showing a 3- to 4-fold reduction in CAT activity. All other mutants had CAT activities comparable with wild type. Mean values for the percent conversion of chloramphenicol to acetylated metabolites are shown in Table 1. CAT assays performed on the cysteine mutants using H9 and U937 lymphoid cells also gave similar results, except that mutant 31 was less active by a factor of ≈4 in H9 cells and less active by a factor of ≈6 in U937 cells than the wild-type provirus (17).

Stable mRNA Expression of Mutant Proviruses. The mRNA expression of each mutant clone was evaluated by Northern blot analysis of total cellular RNA prepared 48 hr after transfection (Fig. 2). The steady-state viral mRNA expression in mutant clones 3, 12, 49, 50, 53, and 4 (data not shown) was comparable, and RNA transcript profiles [9.2, 4.3, 2.0–1.8 kilobases (kb); Fig. 2*A*] were similar to that of the wild-type virus (Fig. 2*C*; lane 1). This suggests that the targeted nucleotide alterations in these mutants did not affect viral function at the steady-state mRNA level. In contrast, the remaining missense mutant clones showed uncharacteristic profiles. Mutant 43 produced two detectable transcripts at 4.3 and 2.0–1.8 kb. Cotransfection with pCV-1, a *tat*-expressing cDNA clone (4), failed to restore expression of the 9.2-kb mRNA on the Northern blots. However, cotransfections with mutant proviruses containing translational stop codons at the N terminus of either *tat* [M*tat*(−)] or *rev* [M*rev*(−)] (8), restored the expression of the 9.2-kb genomic RNA (Fig. 2*B*), possibly through recombination.

All the cysteine missense mutants gave barely detectable levels of viral transcripts (5–10% of wild-type expression),

Table 1. Comparison of biological properties of HIV-1 missense mutants

Clone designation	CAT activity, % conversion	RNA		gag proteins	Virus	Complementation			
		Species	-fold (×)			pCV1	p*tat*	p*rev*	p*tat* + p*rev*
WT	50.5	All (9.2, 4.3, 2, and 1.8 kb)	1	+	+				
3	30.0	All	1	+	+				
4	50.0	All	1	+	+				
12	39.5	All	1	+	+				
25	1.7	1.8 kb	≤0.1	−	−	+	+	−	+
27	1.3	1.8 kb	≤0.1	−	−	+	+	−	+
30	2.1	1.8 kb	≤0.1	−	−	+	+	−	+
31	51.5	3.5 and 1.8 kb	0.2	−	−	−	−	+	+
34	0.9	1.8 kb	≤0.1	−	−	+	+	−	+
37	0.6	3.5 and 1.8 kb	≤0.1	−	−	+	+	−	+
43	12.0	4.3 and 1.8 kb	1	−	−	−	−	−	+
49	33.7	All	1	+	+				
50	43.2	All	1	+	+				
53	42.6	All	1	+	+				

Common features of replication-defective proviruses are (*i*) reduced capacity to trans-activate, (*ii*) altered patterns of stable viral mRNAs and proteins, and (*iii*) absence of virus particles. Numerical designations of the mutant clones referring to position of altered amino acid in *tat* are illustrated in Fig. 1. CAT values are from three independent transfection experiments (variation ±20%). RNA species analyzed by Northern blots were quantitated by densimetric tracings of the hybridized bands; 1×, 1-fold (100% wild type).

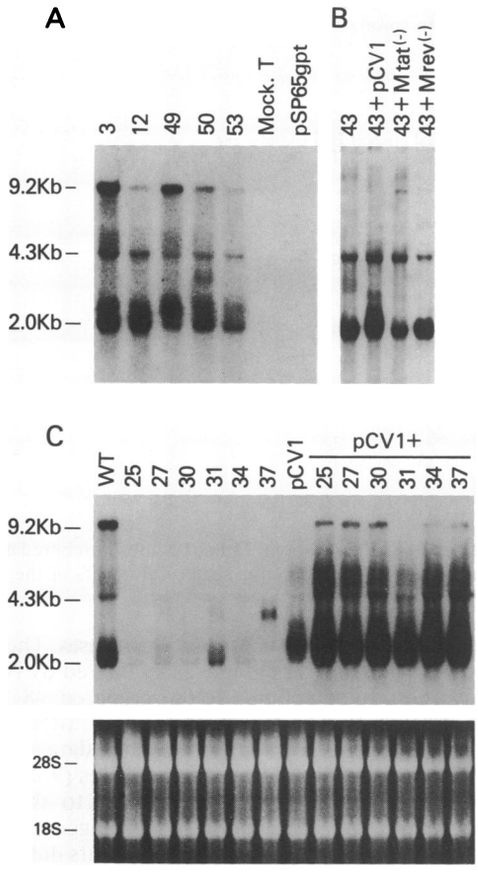

FIG. 2. Northern analysis of wild-type (WT) HXB2 and missense mutant clones. RNA samples derived from the transfectants are indicated above each lane. Numbers 3–53 (*A*, *B*, and *C*) refer to mutant proviruses illustrated in Fig. 1. (*A*) Mock. T, no plasmid; pSP65gpt, plasmid vector with no provirus insert. (*B* and *C*) + indicates cotransfections; for description of pCV1, M*tat*(−), and M*rev*(−), see text. A faint series of bands appears between 4.3 and 6.0 kb in wild-type and mutant clones complemented by pCV-1 (pCV1). These bands have not been characterized and may represent aberrantly initiated or partially spliced mRNA species derived from pCV-1. In the lower portion of *C*, ethidium staining illustrates the total RNA applied to each lane; RNA input is essentially equal in the lanes.

except for mutant 31, which was also able to efficiently trans-activate the homologous LTR in CAT assays (see Table 1). However, mutant 31 did not express the wild-type pattern of viral mRNA. Instead, a major band at 1.8 kb (50% of wild-type level) and a faint band at 3.5 kb were detected. This unusual 3.5-kb transcript was present alone and in greater amounts (≈5-fold) in mutant 37-transfected cells (Fig. 2*C*, left). Complementation of these cysteine mutants by cotransfection with plasmid pCV-1 resulted in the production of normal mRNA size classes and levels, except for mutant 31, which was not complemented by the *tat* gene (pCV-1).

Ability of the Mutants to Produce Protein and Virus. The level of protein expression of the mutant clones was evaluated by RIP assays of extracts from cells that were transfected and metabolically labeled with [^{35}S]methionine. Mutants 3, 4, 12, 49, 50, and 53 expressed protein levels similar to the wild type. Cysteine mutants 25, 27, 30, 31, 34, and 37 did not express detectable structural and core proteins (data not shown). Leucine mutant 43 (Leu → Phe) expressed envelope proteins (gp 160/120) at levels comparable to the wild type but not the core proteins (p25/24 *gag*). Fig. 3 illustrates the protein profiles of representative mutants from each phenotypic group: wild type and mutant 4 (lanes 1 and 2); mutant 43 (lane 3); and wild-type transfectants immunoprecipitated with normal IgG (lane 4), a pattern similar to that seen with the cysteine mutants.

cDNA clones, that carried *tat*, pCV-1 (4), and p*tat* (18) were unable to complement either mutant 31 or mutant 43. Although both mutations lie outside the coding sequence of *rev*, the aberrant RNA profiles (e.g., the absence of a 9.2-kb band) are reminiscent of the rev$^-$ phenotype (5, 8). Therefore, we attempted complementation by a *rev*-expressing cDNA clone (p*rev*) (18). For mutant 31, virus production was restored. Mutant 43, however, required both p*tat* and p*rev* for virus production. Mutant proviruses M*tat*(−) or M*rev*(−) were also able to "complement" mutant 43, resulting in both extracellular and budding virus particles with characteristic wild-type structure (Fig. 4). Moreover, the viruses produced were infectious upon cocultivation with CD4$^+$ H9 lymphocytes and cytopathic when cocultivated with human peripheral blood lymphocytes (data not shown). These observations suggest the possibility of homologous recombination between the mutant proviruses. Additionally, because more than one genomic RNA is packed per virus particle (1), a portion of the virus progeny could contain heterodimer RNA genomes, even at low multiplicity of infection. However, these particles would be defective in the case of M*rev*(−) and mutant 43 because both *tat* and *rev* appear to be required for complementation.

Detection of the *rev* Splice Acceptor Site. The fact that complementation of mutant 43 required both p*tat* and p*rev* suggested that the *rev* gene is also rendered defective by this mutation. In determining the basis of this defect, we located a splice acceptor consensus sequence (CCTTAGG) (19) spanning the mutation site. Previously, it was assumed that *tat* and *rev* were expressed from a common polycistronic mRNA as represented by pCV-1 (4, 5). However, pCV-1 expresses *tat* efficiently and *rev* only poorly (8), suggesting that the two genes may be differentially regulated. Although the polycistronic cDNA clone pCV-1 can complement M*rev*(−) mutant, it does so poorly as compared with p*rev* (M.R.S., unpublished work). This is also supported by cotransfection experiments in which mutant 43 is complemented by a combination of p*tat* and p*rev* but not with pCV-1. Presumably, the AUG initiation codon for *rev* is not used effectively in pCV-1, as it lies downstream from the *tat* AUG. Initiation of translation occurs preferentially at the AUG codon closest to the 5′-end of the mRNA (20).

To test the possibility that *rev* is expressed from a separate mRNA, using this putative splice acceptor site, we performed

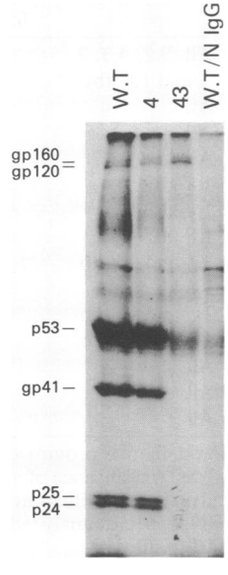

FIG. 3. Analysis of structural proteins of wild-type and mutant proviruses. Labeled proteins from transfected cells were immunoprecipitated with HIV-1-positive sera. The source of lysate used for RIP is indicated above each lane; see Fig. 1 for mutant key. W.T, wild type (HXB2). N IgG, normal human immunoglobulin.

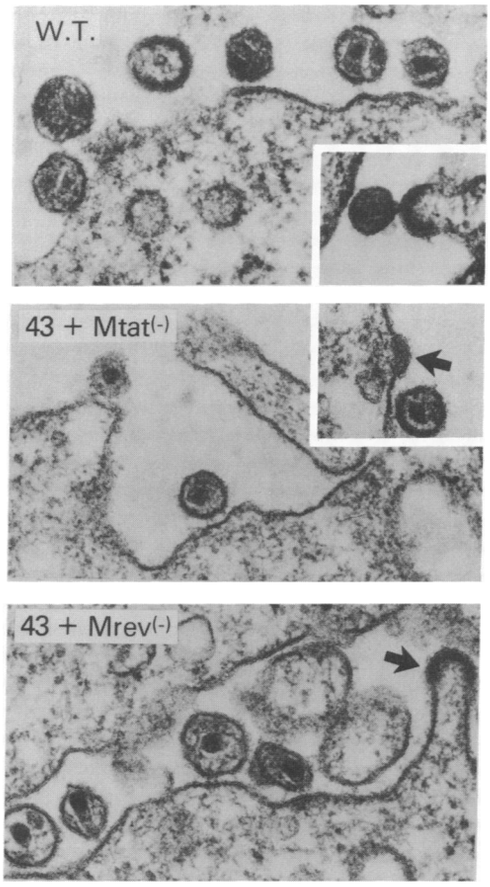

FIG. 4. Electron microscopic visualization of virus particles in transfected cells. COS-1 cells transfected with wild-type pHXB2 (W.T.) or mutant 43 together with either *tat*(−) [43 + M*tat*(−)] or *rev*(−) [43 + M*rev*(−)] mutant proviruses. Cells were fixed with 1% glutaraldehyde and processed for electron microscopy 48 hr after transfection. *Inset* shows and arrows point to budding particles. (×81,000.)

S1 nuclease analysis of RNAs from wild-type and mutant proviruses with an oligonucleotide probe depicted in Fig. 5*A*. The major products of S1 digestion are a fully protected 60-nucleotide (nt) band representing RNA reading through this region and a cleavage product of 34 nt. This cleavage corresponds to the 3′ border of the intron–exon junction predicted for the rev mRNA. According to the ↓GT...AG↓ rule (19), the predicted S1 product is 31–34 nt based on cleavage within 4 nt upstream of the ...AG↓ acceptor. Fig. 5*B* shows that the 60- and 34-nt protected bands were detected using RNA from HIV-1-infected H9 cells (lane 6), wild-type HXB2, and M*rev*(−) COS-1 transfectants (lanes 8 and 11). Mutant 43 failed to show both 60- and 34-nt S1 products. The absence of the 34-nt band supports the hypothesis that the *rev* defect is due to alteration of the proposed splice acceptor. The loss of the fully protected 60-nt band may be due to the lower level of tat mRNA (Table 1) and the absence of genomic length RNA (Fig. 2*B*). Because the 4.3-kb env mRNA is expressed by mutant 43, the absence of a protected band using this particular probe suggests that env mRNA is generated via a splice acceptor outside this region. Using a labeled synthetic oligonucleotide complementary to the region spanning nt 5562–5611, we detected equal levels of fully protected probe in mutant 43 as compared with wild type (M.R.S., unpublished observation). Although it previously was assumed that *env* (22), *tat* (4), and *rev* (5) use the same splice acceptor (nt 5356), we now propose that *env* uses the splice acceptor previously ascribed to *nef* (nt 5557) (4) and *rev*

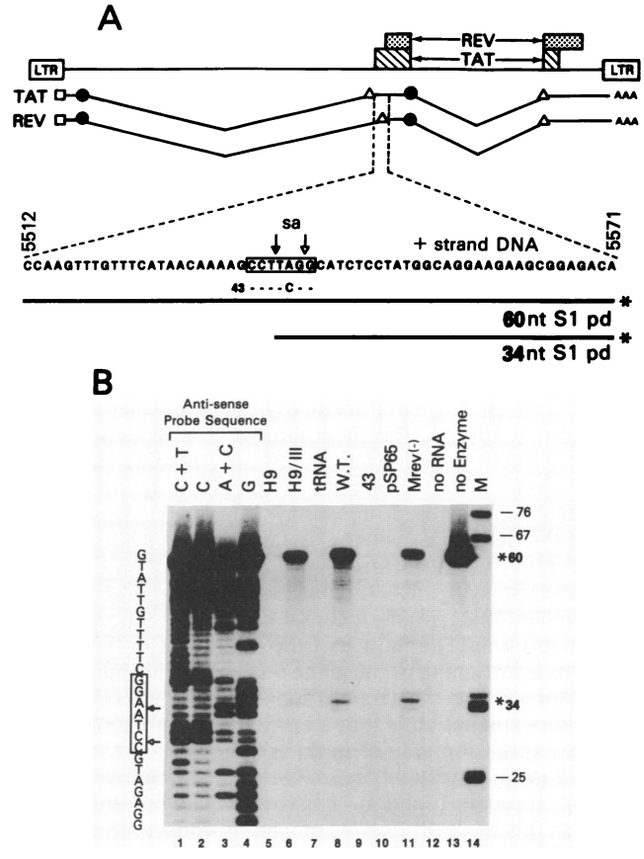

FIG. 5. (*A*) Schematic representation of *tat* and *rev* splicing patterns. Positions of coding exons are indicated (top line) by hatched and dotted boxes for *tat* and *rev*, respectively. Transcription initiation site, splice donor sequence, and acceptor sequences are indicated by square, closed circle, and open triangle, respectively. The origin containing the putative *rev* splice acceptor (sa) site is indicated by dashed lines. The DNA sequence is shown, and the putative splice acceptor is boxed. Also shown are the S1 nuclease products (pd) (60 and 34 nt). The splice site detected experimentally is indicated in the sequence by closed arrow; the theoretical acceptor site is indicated by open arrow. Position of the nucleotide change in mutant 43 is indicated beneath the box. (*B*) S1 nuclease analysis of RNAs from mutant and wild-type HIV-1 transfectants. Lanes 1–4 represent Maxam and Gilbert (21) sequence ladder generated by cleavage of the 60-nt probe; hybridized RNAs were as indicated above each lane.

uses the splice acceptor at nt 5540. The predominance of the 60-nt band in wild type represents the relative abundance of stable mRNA not spliced within this region. We also used a synthetic oligonucleotide probe spanning the proposed splice junction of *rev* for S1 nuclease analysis of H9/III RNA. The fully protected band representing rev mRNA was less by a factor of ≈10 than the cleavage product corresponding to the messages not spliced within this region (data not shown), consistent with the lower abundance of rev mRNA depicted in Fig. 5*B*.

DISCUSSION

We used site-directed mutagenesis to generate missense mutations within an HIV-1 provirus genome. This approach in combination with various phenotypic analyses enabled us to ascertain the importance of targeted amino acid residues in the *tat* gene product. We observed that mutations within the N-terminal region of *tat* had no effect on *tat* activity or virus replication. It is possible that the overall structure of *tat* was unaffected by the changes.

Within the central cysteine-rich region of *tat*, several functionally defective mutants were examined. Amino acid substitutions in each of the six targeted cysteine residues resulted in defective proviruses. Five of the six cysteine mutants lost *tat* activity, and virus production was complemented by a tat cDNA clone. At present, we cannot determine whether these mutations in cysteines affect the functional activity of *tat per se*, or its stability or subcellular localization, which, in turn, lowers *tat* activity. Because *tat* positively regulates its own expression from the proviral LTR, defective tat proteins are not synthesized in measurable quantities for this evaluation. The cysteine-rich region was thought to form a metal-binding finger because of its structural similarity to analogous regions of known metal-binding regulatory proteins (23). Recently, tat protein has been reported to form a metal-mediated dimer distinct from traditional metal-linked finger structures (24). Whether or not the tat protein functions through such binding properties [either alone or in combination with cellular factor(s)] is an important unanswered question. Mutant 31 was unique among the defective cysteine mutants in that it was positive for *tat* activity but negative for virus production (Table 1). The *tat*-expressing cDNA clones (pCV-1 and p*tat*) failed to complement this mutant, whereas complementation with *rev* produced virus. The nature of the *rev* defect in this mutant provirus is intriguing because the position of the mutation lies upstream of the *rev* translational initiation codon. One plausible explanation is that a cis-acting element(s) required for *rev* expression resides in this region.

Mutations in the highly basic C-terminal Arg/Lys residues did not abrogate function. This result was not anticipated, because this basic stretch is completely conserved among all HIV-1 isolates and therefore was predicted to be an important functional domain of *tat*. Because single substitutions in this region did not abolish *tat* activity, it would be of interest to test clones containing multiple mutations in this area.

An additional mutation within the C-terminal region of *tat* (mutant 43) resulted in a defect in *rev* activity, even though the mutation was positioned upstream of the *rev* coding sequence; this mutant had reduced *tat* activity and expressed aberrant viral transcripts. Complementation by the combination of both tat and rev cDNA clones showed the dual defect of this mutant provirus. In studying the basis for the *rev* defect, we located a potential splice acceptor site by DNA sequence homology and confirmed the use of this splice acceptor by S1 nuclease analysis. Our results indicate that this splice acceptor site is used to generate rev mRNA and that tat and rev proteins are translated from different mRNA species. Unlike M*rev*(−), mutant 43 expresses levels of 4.3-kb mRNA similar to wild type. It is possible that a very low level of rev protein is produced from alternate spliced mRNA (e.g., the tat mRNA), and that the gag mRNA (9.2 kb) is more sensitive to *rev* depletion than the env mRNA. This hypothesis is not surprising because the primary effect of *rev* is to promote the relative accumulation of unspliced mRNA at the expense of spliced mRNA (5, 8) and gag mRNA would have to be protected from two splicing events as opposed to only one for env mRNA.

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