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A point mutation in the HIV-1 Tat responsive element is associated with postintegration latency

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ABSTRACT **Study of the mechanism of HIV-1 postintegration latency in the ACH2 cell line demonstrates that these cells failed to increase HIV-1 production following treatment with exogenous Tat. Reasoning that the defect in ACH2 cells involves the Tat response, we analyzed the sequence of tat cDNA and Tat responsive element (TAR) from the virus integrated in ACH2. Tat cDNA sequence is closely related to that of HIV_{LAI}, and the encoded protein is fully functional in terms of long terminal repeat (LTR) transactivation. Cloning of a region corresponding to the 5′-LTR from ACH2, however, identified a point mutation ($C^{37} \rightarrow T$) in TAR. This mutation impaired Tat responsiveness of the LTR in transient transfection assays, and the measured defect was complemented in cells that had been treated with tetradecanoyl phorbol acetate or tumor necrosis factor type α (TNF-α). A compensatory mutation in TAR ($G^{28} \rightarrow A$), designed to reestablish base pairing in the TAR hairpin, restored wild-type Tat responsiveness. When the ($C^{37} \rightarrow T$) mutation was introduced in an infectious clone of HIV-1, no viral production was measured in the absence of TNF-α, whereas full complementation was observed when the infection was conducted in the presence of TNF-α or when a compensatory mutation ($G^{28} \rightarrow A$) was introduced into TAR. These experiments identify a novel mutation associated with HIV-1 latency and suggest that alterations in the Tat–TAR axis can be a crucial determinant of the latent phenotype in infected individuals.**

Although active viral replication occurs during all phases of HIV-1 infection in patients (1, 2), evidence has been presented that a large proportion of HIV-1-infected cells carry the virus in an latent state (3–7). This state is characterized by the presence of integrated viral DNA and low levels of viral RNA or protein and has been coined postintegration latency (8, 9). The mechanism of postintegration latency and its relevance to HIV pathogenesis are subjects of intense study; however, no description at the molecular level has emerged.

The ACH2 cell line was established by rescuing and cloning A3.01 cells surviving an acute infection with HIV_{LAI} and contains a single integrated copy of HIV-1 (10, 11). Under basal conditions, the virus integrated in ACH2 cells (called HIV_{ACH2} hereafter) shows very low basal transcription and a predominance of multiply spliced transcripts encoding HIV-1 regulatory proteins (12). Upon stimulation with tumor necrosis factor type α (TNF-α) or phorbol esters, an increase in transcription is noted, followed by the appearance of unspliced transcripts encoding viral structural proteins and viral production (11–13). The molecular mechanism underlying the suppression of transcription under basal conditions in ACH2 cells is unclear. It has been suggested that the site of integration of the virus in the cellular genome and its local chromatin environment might play a role in causing latency (14, 15). In agreement with this model, we have demonstrated that a chromatin disruption occurs in the promoter of HIV-1 during transcriptional activation in ACH2 cells (16, 17). However, this chromatin disruption is not specific for the ACH2 cells and has been observed in all cell lines examined, independent of the site of integration of the virus (16, 17). On the other hand, the virus integrated in the ACH2 cells is uniquely unresponsive to exogenous Tat (18, 19), suggesting that the Tat–Tat responsive element (TAR) axis might be altered in $HIV\text{-}1_{ACH2}$. Here, we report that a single point mutation located in the TAR accounts for postintegration latency in the ACH2 cell line.

MATERIALS AND METHODS

Cell Culture. All cell lines were obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and grown as reported (16, 17).

Cloning of ACH2 *tat* cDNA. RNA was purified from tetradecanoyl phorbol acetate (TPA)-treated ACH2 cells (10 nM for 16 hr) using Trizol (GIBCO/BRL). Oligo(dT)-primed cDNA synthesis was performed using the 1st-Strand cDNA synthesis kit (CLONTECH). This cDNA was used as a substrate in PCRs using oligonucleotide primers with the following sequence: sense, 5′-ACGT**GGATCC**TTACTCAACAGAGGAGAGCAAGAA$^{=nt5409}$-3′ and antisense, 5′-A$^{=nt8080}$GATCGTCCCAGATAAGTGCTAA**GGATCC**GTTCA-3′ (a new *Bam*HI site, introduced to facilitate cloning, is indicated in boldface type). The PCR fragment was gel-purified, digested with *Bam*HI, and ligated into the unique *Bam*HI site of pREP9 (Invitrogen). The sequences of 10 independent transformants were determined using an automated sequencer and the Prism kit (Applied Biosystems). The nucleotide sequence all 10 clones were identical, in agreement with the fact that the ACH2 cell line contains a single integrated copy of HIV-1. The same strategy was used to clone the two-exon form of *tat* from the vector pCV1 (20) with the following oligonucleotides: sense, 5′-ACGT**GGATCC**TTACTCAACAGAGGAGAGCAAGAA-3′ and antisense, 5′-CGC**GGATCC**GATGGAG CCAGTAGATCCTAGACTAGAGC-3′ (boldface type indicates *Bam*HI sequence). This vector is designated pREP9/tat2exon.

Cloning of ACH2 Provirus Long Terminal Repeat (LTR). DNA purified from exponentially growing ACH2 cells, as described (17), was used in PCR with the following primers: sense, 5′-GC**CTGCAG**TGGAAGGGCTAATTCACTCCCAACG (nt 1–25, HIV_{LAI}) and antisense, 5′-GC**TCTAGA**CTC-

Abbreviations: TNF-α, tumor necrosis factor type α; TAR, Tat responsive element; TPA, tetradecanoyl phorbol acetate; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; $TCID_{50}$, tissue culture 50% infective dose.

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TCTCCTTCTAGCCTCCGC (nt 791–769, HIV_{LAI}). These primers contain added restriction sites for *Pst*I (sense) and *Xba*I (antisense) restriction enzymes (highlighted in boldface type above) to allow directional cloning of the PCR product. The PCR fragment cloned into pCAT-BASIC (Promega) was digested with *Pst*I and *Xba*I. Several clones were selected, sequenced on both strands, and found to have identical sequences. This plasmid is designated $pLTR_{ACH2}$-CAT. The same strategy was used to clone the LTR of HIV_{LAI} using pLAI2 as a substrate in the PCR. This plasmid is designated $pLTR_{LAI}$-CAT.

DEAE-Dextran Transient Transfections. Transient transfection assays were carried as described (21). Transfection experiments were repeated three or more times in duplicate or triplicate. Transfection efficiency varied by <20% from experiment to experiment.

Site-Directed Mutagenesis. Mutagenesis was performed using double-stranded plasmids and a selection oligonucleotide with the Transformer kit (CLONTECH). Two different mutations, corresponding to the changes ($C^{37} \rightarrow T$) and ($G^{28} \rightarrow A + C^{37} \rightarrow T$) were introduced into $pLTR_{LAI}$-CAT using the following oligonucleotides: 5′-GAGCCTGGGAGTTCTCTGGCTAAC-3′,5′-CCAGATTTGAACCTGGGAGTTCTCTGGCTAAC-3′ (mutations in boldface type), and a selection oligonucleotide changing a unique *Bam*HI site in $pLTR_{LAI}$-CAT into an *Sph*I site, 5′-CATGTCTGCATGCCCCGGAATTC-3′. One mutation was introduced in $pLTR_{ACH2}$-CAT ($T^{37} \rightarrow C$) with the oligonucleotide 5′-GAGCCTGGGAGCTCTCTGGCTAAC-3′ and the same selection oligonucleotide as above.

Generation of Infectious Provirus. A *Nar*I–*Sph*I fragment containing the complete HIV-1 LTR was obtained after partial digestion with *Nar*I and complete digestion with *Sph*I of pNL4–3 (22). This fragment was subcloned into pUC19 digested with *Nar*I and *Pst*I and was used for site-directed mutagenesis using the Transformer protocol (CLONTECH). Mutated clones were identified using cycle-sequencing (Applied Biosystems) and were fully resequenced between *Mro*I and *Bss*HII after identification. An *Mro*I–*Bss*HII fragment corresponding to nt 308–711 was purified and introduced into the unique *Mro*I–*Bss*H2 sites of pILIC19 (23, 24), thereby reconstituting a circularly permutated HIV-1 clone. As a control, an unmutated fragment was purified from pNL4-3 and cloned into pILIC19. Infectious stocks were generated from these clones after *Bam*HI digestion, self-ligation, and transfection into SupT1 cells. Infections were carried out by incubating 10^6 cells with virus aliquots corresponding to different amounts of p24 antigen at 37°C for 2 hr in 100 μl. Cells were then washed three times and diluted in standard culture medium and grown under normal conditions.

RESULTS

Effect of Exogenous Tat on HIV-1 Expression in ACH2 Cells. Because Tat is a critical regulator of HIV-1 transcription and Tat-defective viruses exhibit a latent phenotype, we first examined the ability of exogenous Tat to induce viral expression in ACH2 and U1 cells. Purified Tat protein (25), which is taken up by living cells (26, 27), was incubated with both cell lines at different doses, and virus production was estimated by measuring p24 antigen in the supernatant of both cell lines. Whereas Tat induced HIV-1 expression in the U1 cells at doses as low as 185 ng/ml, no effect of Tat was observed on viral expression in the ACH2 cells under the same conditions (Fig. 1). This experiment clearly indicates that distinct mechanisms of HIV-1 latency are at play in these two cell lines and that the Tat response is defective in the ACH2 cells as reported by others (14, 15, 18, 19, 29). Tat action could be ineffective in ACH2 cells due to the site of integration of the provirus, as proposed (14, 15), or as a result of a mutation in one of the components necessary for Tat activity. These include: (*i*) the gene for Tat itself, as some *tat* mutations exhibit a dominant negative phenotype (30, 31); (*ii*) cellular cofactors necessary for Tat activity; and (*iii*) the TAR, where Tat and one or several cofactors bind.

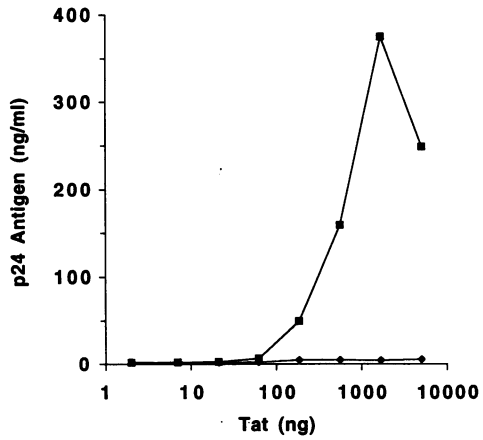

FIG. 1. Effect of exogenous Tat on HIV-1 production in ACH2 and U1 cells. Each cell line (10^6 cells) was incubated with purified Tat for 24 hr (28), and viral production was estimated by measuring secreted p24 antigen in supernatants. ACH2, ◆; and U1, ■.

Sequence and Function Analysis of *tat* Gene from HIV_{ACH2}. First, we examined the sequence of the *tat* gene from ACH2 provirus and the function of the Tat protein that it encodes. A fragment corresponding to the two-exon form of the *tat* gene was amplified by PCR using cDNA obtained from the ACH2 cells and cloned downstream of the RSV promoter. Analysis of several clones showed that the Tat protein from the ACH2 provirus is closely related to the LAI virus, as expected, because this virus stock was used to generate the ACH2 cell line. Three amino acid changes were detected when compared with Tat_{LAI}: $T^{39} \rightarrow M$, $G^{61} \rightarrow D$, and $P^{77} \rightarrow T$. Functional analysis was performed by cotransfection of this expression vector and of a reporter vector containing the LTR of HIV-1 driving the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene ($pLTR_{LAI}$-CAT). This experiment showed that the ACH2 Tat protein exhibited normal transactivation of the HIV-1 LTR when compared to the activity of the HIV-1_{LAI} Tat (Table 1). In addition, high CAT activity was detected cells after transfection of the same vector in ACH2 cells in the absence of an exogenous tat expression vector, demonstrating that ACH2 cells contain an endogenous functional Tat activity (data not shown). These results demonstrate that the Tat protein from the ACH2 provirus is functionally intact and that cellular components necessary for Tat action are intact in ACH2 cells. These results point to a defect at the level of TAR in the virus integrated in ACH2 cells.

Sequence Analysis of LTR from HIV_{ACH2}. To examine this possibility, we determined the sequence of the LTR of the ACH2 provirus. A region corresponding to the first 791 nt of the provirus was amplified by PCR using ACH2 DNA as a

Table 1. Comparison of the transactivating activity of Tat_{LAI} with Tat_{ACH2}

Tat Plasmid	Tat_{LAI}	Tat_{ACH2}
0 μg	10 ± 0.1	10 ± 0.1
0.2 μg	222 ± 5	298 ± 9
1.0 μg	474 ± 9	495 ± 20

A3.01 cells were cotransfected with increasing concentrations of expression vectors for Tat_{LAI} or Tat_{ACH2} and with a reporter plasmid containing the HIV-1 LTR driving the CAT gene. Results are the average of three determinations (± SEM) from a representative experiment.

substrate. The amplified fragment was cloned upstream of the CAT gene in pCAT-BASIC. Alignment of the sequence from several clones with the sequence of HIV_{LAI} showed six differences between the nucleotide sequences of the two viruses: $G^{-347} \rightarrow A$, $C^{-335} \rightarrow T$, $T^{+24} \rightarrow C$, $C^{+37} \rightarrow T$, $G^{+122} \rightarrow A$, and $T^{+201} \rightarrow C$. Most of these mutations are found in other HIV-1 viruses and are probably of little functional consequence. However, a mutation in the TAR region at nucleotide +37 (C → T) is not found in other HIV-1 isolates and is present in a region critically important for Tat action, immediately after the loop of the TAR hairpin (Fig. 2, compare $pLTR_{LAI}$-CAT with $pLTR_{LAI}$ ($C^{37} \rightarrow T$) CAT). This mutation is not an artifact of PCR amplification and was confirmed by Southern blot analysis of ACH2 DNA as it causes the loss of a *Sac*I–*Ban*II restriction site (data not shown). In addition, the mutation reported was observed in ACH2 cells obtained from two independent sources, and it thus was not restricted to one specific clone of ACH2 cells.

A TAR Point Mutation Affects Tat-Responsiveness in Transient Transfection Assays. To test the effect of this mutation on HIV-1 LTR activity and its Tat responsiveness, we compared the activity of a wild-type construct, $pLTR_{LAI}$-CAT, which contains the complete HIV-1 LTR (nt 1–791) driving the CAT gene, with the activity of the same plasmid carrying distinct mutations: $pLTR_{LAI}$ ($C^{37} \rightarrow T$) CAT contains the LTR with a mutation similar to the mutation found in the ACH2 cells; $pLTR_{LAI}$ ($G^{28} \rightarrow A$ and $C^{37} \rightarrow T$) CAT contains the ACH2 mutation ($C^{37} \rightarrow T$) and an additional mutation ($G^{28} \rightarrow A$) designed to restore base pairing in the TAR stem loop structure; $pLTR_{ACH2}CAT$ contains the LTR from the ACH2 virus; $pLTR_{ACH2}$ ($T^{37} \rightarrow C$) CAT contains the LTR from the ACH2 virus with a correction of the TAR mutation (Fig. 2). These five constructs were cotransfected in A3.01 cells with a Tat expression vector (pREP9/Tat2exon) or a control vector (pREP9). In the absence of Tat, no significant differences were observed between the activities of the different plasmids (data not shown). In the presence of Tat, CAT activity was 2- to 3-fold lower when a mutation similar to that present in ACH2 provirus ($G^{28} \rightarrow A$) was present in the construct (Fig. 3, compare lanes 2 and 1, 4 and 5). This defect was corrected when a compensatory mutation was introduced in the RNA hairpin to reestablish base pairing (Fig. 3, lane 3). This defect in CAT expression was totally corrected when cells were treated with TPA (Fig. 3) or TNF-α (data not shown), in agreement with the fact that defective expression of HIV-1 in ACH2 cells is corrected by TNF-α or TPA treatment.

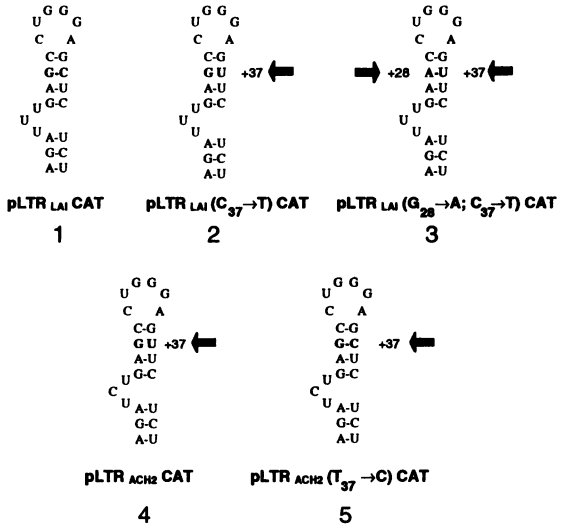

FIG. 2. Sequence of TAR mutants. A schematic representation of the folded TAR region (32, 33) corresponding to nt +20 to +42 is shown for different mutants used in this study. Numbers (1–5) are assigned to each mutation and are used in subsequent figures.

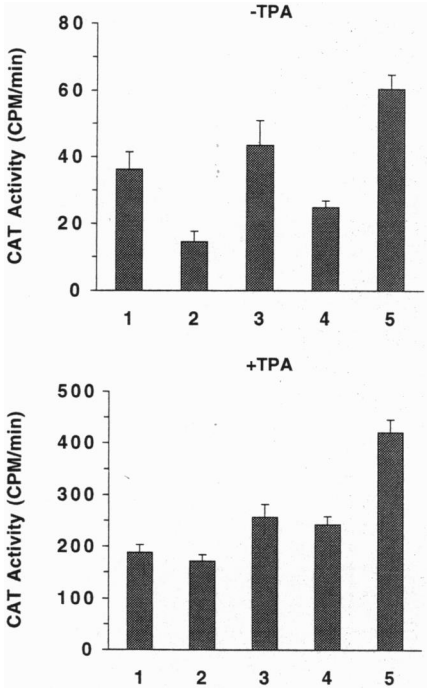

FIG. 3. A TAR mutation impairs Tat responsiveness of the HIV-1 LTR. A3.01 cells were transiently cotransfected with an expression vector for *tat* (pREP9/tat2exon) in the absence of presence of TPA (−TPA or +TPA). Two independent transfections were performed in duplicate or triplicate, and results were averaged. Plasmid 1, $pLTR_{LAI}$-CAT; plasmid 2, $pLTR_{LAI}$ ($C^{37} \rightarrow T$) CAT; plasmid 3, $pLTR_{LAI}$ ($G^{28} \rightarrow A$; $C^{37} \rightarrow T$) CAT; plasmid 4, $pLTR_{ACH2}$-CAT; and plasmid 5, $pLTR_{ACH2}$ ($T^{37} \rightarrow C$) CAT.

A TAR Point Mutation Causes a Latent Phenotype in an Infectious Provirus. To determine the effect of the $C^{37} \rightarrow T$ mutation in the context of an infectious provirus, site directed mutagenesis was performed on the infectious clone pILIC (23, 24). Several infectious HIV-1 molecular clones were generated with LTR sequences identical to those tested in transient transfection assays. These constructs were transfected in A3.01 cells and viral production was measured in supernatants by RT assay. In the absence of TNF-α, low or no detectable virus production was observed as late as 30 days after transfection of the molecular clone containing the ACH2 LTR sequences or a single point mutation ($C^{37} \rightarrow T$) in the context of $HIV\text{-}1_{LAI}$ (Fig. 4). In contrast, vigorous virus production was detected with HIV_{LAI}, with HIV_{LAI} ($G^{28} \rightarrow A$; $C^{37} \rightarrow T$) and with HIV_{ACH2} ($T^{37} \rightarrow C$) (Fig. 4). Remarkably, when transfected cells were treated with TNF-α, viral expression was observed with HIV_{LAI}($C^{37} \rightarrow T$) and with HIV_{ACH2} to the same level as wild-type virus with slightly delayed kinetics (Fig. 4, +TNF-α). In separate experiments, we showed that TNF-α treatment could reactivate viral expression as late as 20 days after transfection (data not shown).

To test the effect of these mutations after infection, viral stocks were generated after transfection of all five viral molecular clones in TNF-α-treated cells. Virus stocks were pelletted and washed three times by centrifugation to eliminate TNF-α and their infectivity measured using a tissue culture 50% infective dose ($TCID_{50}$) assay. Jurkat cells were infected with different amounts of p24 antigen viral equivalents and culture supernatants were assessed 2 weeks after infection for reverse transcriptase activity. The mininum amount of virus necessary for infection of 50% of wells (reverse transcriptase activity >50 cpm/μL) is plotted in Fig. 5. A point mutation in TAR ($C^{37} \rightarrow T$) in HIV_{LAI} caused a 100-fold decrease in

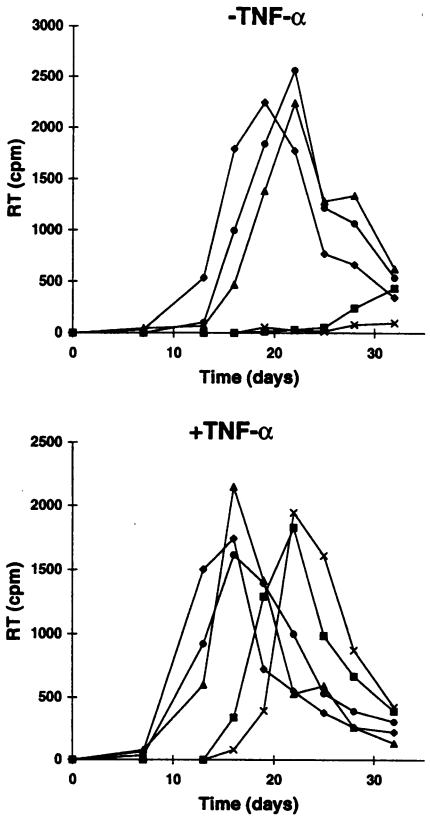

FIG. 4. Effect of TAR mutations on HIV-1 replication in lymphoid cells. Infectious molecular clones of HIV containing the same LTR sequences as the constructs described in Fig. 3 were transfected into A301.1 cells and culture supernatants harvested after different intervals (0–30 days) were assayed for reverse transcriptase activity. HIV_{LAI}, ◆; HIV_{LAI} ($C^{37} \rightarrow T$), ■; HIV_{LAI} ($G^{28} \rightarrow A$; $C^{37} \rightarrow T$), ▲; HIV_{ACH2}, ×; and HIV_{ACH2} ($T^{37} \rightarrow C$), ●.

infectivity (from 0.275 ng of p24 antigen to 27.5 ng; compare virus 2 with 1). Correction of base pairing in TAR by a compensatory mutation ($G^{28} \rightarrow A$) caused a partial restoration of infectivity (10-fold increase) to a $TCID_{50}$ of 2.75 ng of p24 antigen. Similarly, correction of the ($C^{37} \rightarrow T$) mutation present in the ACH2 TAR region caused an 18-fold increase in infectivity ($TCID_{50}$= 0.275 versus 5 ng of p24 antigen; Fig. 5, compare lanes 5 and 4). These results are consistent with those obtained after transfection assays and demonstrate that the TAR mutation present in HIV_{ACH2} results in a dramatic decrease in infectivity.

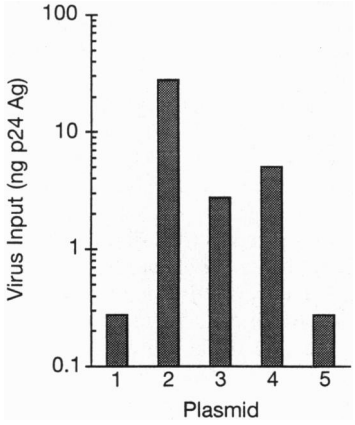

FIG. 5. Effect of TAR mutations on HIV-1 infectivity. Virus stocks were generated after transfection of pILIC and pILIC containing TAR mutations in the presence of TNF-α. Viruses were pelleted and washed three times to remove TNF-α and used to infect A3.01 cells with different doses of p24 antigen. Supernatants were harvested after 14 days, and reverse transcriptase activity were measured. The graph depicts the $TCID_{50}$ in pg of p24 antigen for each of the viruses. 1, HIV_{LAI}; 2, HIV_{LAI} ($C^{37} \rightarrow T$); 3, HIV_{LAI} ($G^{28} \rightarrow A$; $C^{37} \rightarrow T$); 4, HIV_{ACH2}; and 5, HIV_{ACH2} ($T^{37} \rightarrow C$).

DISCUSSION

We have characterized the two components of the Tat–TAR axis in the ACH2 cell line, a model of postintegration latency. The sequence of the Tat protein encoded by the virus integrated in the ACH2 cell line (HIV_{ACH2}) is closely related to the sequence of HIV_{LAI} Tat. The HIV_{ACH2} Tat protein is functionally intact when assayed in transient transfection assays. The R region in the LTR of HIV_{ACH2} contains a unique mutation close to where several transcriptional regulatory elements have been mapped, both at the DNA and RNA level. This mutation significantly reduces the ability of Tat to transactivate the HIV promoter when introduced in an LTR-CAT construct. When introduced in an infectious clone of HIV-1, this mutation is associated with a latent phenotype and a dramatic decrease in infectivity (100-fold), which can be alleviated by TPA or TNF-α treatment, thereby reproducing the phenotype observed for the ACH2 cells.

The mutation found in HIV_{ACH2} is located in a region of TAR crucial for transactivation by Tat and disrupts base pairing at the top of the TAR hairpin. This region of TAR is highly structured and is situated between the binding sites for Tat which binds to the bulge (refs. 34–36; Fig. 2) and for a cellular cofactor which binds to the loop (refs. 37 and 38; Fig. 2). A G-C base pair is replaced by a G-U which can also pair in RNA, but with a stability lower than that observed for either G-C or A-T. This change could account for local conformational change resulting in a decrease binding of Tat, its cofactor, or both.

A discrepancy was noted when comparing the defect caused by this mutation in transient transfection assays (2- to 3-fold suppression; see Fig. 3) with its effect on virus production, where this mutation essentially appeared lethal (Fig. 4). A similar discrepancy has been observed in a previous study examining the effect of TAR mutation in virus infection assays (39). This discrepancy could reflect the presence of a threshold factor in *tat* activity *in vivo* or could be due to the relative inadequacy of transient transfection assays to measure Tat activity in a physiologically relevant manner. This discrepancy may also underscore the role played by chromatin in HIV-1 transcriptional regulation as demonstrated (16, 17) and, in particular, suggests that Tat might be particularly important in alleviating the repressive effects of chromatin on the HIV-1 promoter.

We have not completely excluded the possibility that the mutation observed in the ACH2 LTR exerts its effect by disrupting the binding of a transcription factor at the DNA level and not at the RNA level. However, the fact that Tat transactivation of the mutant TAR is corrected by a compensatory mutation at nt +28 (G → A) both in transfection and infection assays strongly argues for the role of a secondary structure in this region and consequently for an effect at the RNA level. We have compared the ability of DNA fragment corresponding to the wild-type sequence and to the ACH2 sequence in gel-shift assays and have not detected any differences in factor binding between the two probes (S.E. and E.V., unpublished observation). These data argue against a mechanism mediated at the DNA level.

The results reported here provide a molecular explanation for previous observations that the virus integrated in ACH2 cells is unresponsive to Tat, either provided exogenously or provided by a superinfecting virus (14, 15, 18, 19). It has been suggested that this unresponsiveness is secondary to the site of

integration of the virus in the cellular genome (14). However, our results demonstrate that the TAR mutation is sufficient to explain the Tat unresponsiveness of HIV_{ACH2}. In terms of pathogenesis, these findings illustrate a new mechanism of HIV-1 latency and point to the crucial role played by the Tat–TAR axis in this instance. Because our experiments (Fig. 1) and observations by others (15, 18, 19, 40) have suggested that Tat function is deficient in U1 cells, we have cloned the cDNAs encoded by the two proviruses integrated in U1 cells and have found that both cDNAs contain mutations resulting in transactivation-defective *tat* proteins (S.E. and E.V., unpublished data).

These results suggest that an interruption of the Tat–TAR axis might be the basis for postintegration latency in HIV-1-infected individuals. In fact, studies examining the sequence of the *tat* gene in patients have demonstrated that the *tat* genes of a large proportion of viruses contain mutations that significantly affect Tat transactivating activity (41). This fact raises the possibility that accumulation of proviruses carrying mutations in the Tat–TAR axis might account for the progressive rise in viral burden observed throughout the course of the disease. The accumulation of defective viruses could in turn play a significant role in pathogenesis, as viral products have been found to be expressed at low levels in these cells and could potentially affect the differentiated immune functions of these cells.

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