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A Critical Role for the TAR Element in Promoting Efficient Human Immunodeficiency Virus Type 1 Reverse Transcription

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The regulation of human immunodeficiency virus type 1 (HIV-1) gene expression is dependent on the transactivator protein Tat and an RNA element extending from the transcription initiation site to +57 known as TAR. TAR forms a stable RNA secondary structure which is critical for high levels of HIV-1 gene expression and efficient viral replication. Using a genetic approach, we isolated HIV-1 mutants in TAR that were competent for high levels of gene expression but yet were markedly defective for viral replication. Single-cycle infections with these viruses demonstrated that they were defective in the initiation of reverse transcription. Additional mutational analysis revealed a variety of other HIV-1 TAR mutants with the same defective phenotype. Thus, in addition to the well-characterized role of the primer binding site, other RNA elements within the HIV-1 genome are also critical in the regulation of reverse transcription. These studies demonstrate that HIV-1 TAR RNA is a key regulator of the reverse transcription and illustrate how a unique RNA structure can modulate diverse regulatory processes in the HIV-1 life cycle crucial for efficient viral replication.

Like all retroviruses, the human immunodeficiency virus type 1 (HIV-1) uses the combined activities of the virus-encoded heterodimeric reverse transcriptase to synthesize proviral DNA from the RNA genome (5, 36, 52). Reverse transcription is a tightly regulated process that is initiated by the action of reverse transcriptase which in conjunction with a cell-derived tRNA molecule binds to RNA sequences known as the primer binding site (PBS) (44). The last 18 nucleotides of $tRNA_3^{Lys}$, which are complementary to the HIV-1 RNA PBS sequences (23), function to prime HIV-1 DNA synthesis by initiating the production of minus-strand strong-stop DNA (21, 53, 58). The full-length double-stranded DNA molecule is generated in a multiple-step process that is initiated by a jump of the minus-strand strong-stop DNA to the 3′ end of the RNA genome (28, 29). The reverse transcription process is not completely understood, and questions still remain concerning the role of additional viral and cellular regulatory proteins and the involvement of RNA elements other than the PBS.

HIV-1 mutants which change the PBS to sequences which bind tRNA molecules other than $tRNA_3^{Lys}$ replicate with reduced kinetics compared with wild-type virus and rapidly revert to the native PBS sequences (15, 38, 47, 55, 56). Although the mechanisms responsible for the observed reversions are unknown, the rapid reversion rates suggest that the native HIV-1 PBS is maintained because of stringent selective pressure. Both UV cross-linking and gel retardation analysis indicate that highly specific interactions between HIV-1 reverse transcriptase and its primer tRNA (6) can induce conformational changes in the heterodimeric reverse transcriptase enzyme (48, 61). Studies with other retroviruses suggest that RNA sequences outside the PBS are also involved in the control of reverse transcription. For example, the initiation of reverse transcription in Rous sarcoma virus is decreased by mutations that alter the structure of a complex RNA stem-loop structure formed by sequences flanking the PBS (1, 12, 13). Among different retroviruses, it has been demonstrated that the PBS, the flanking RNA sequences, the specific tRNA primer utilized, and the reverse transcriptase are not interchangeable (2). Thus, while the steps involved in reverse transcription can be generalized for all retroviruses, efficient initiation of this process is restricted to selective components that comprise the initiation complex.

HIV-1 utilizes multiple RNA secondary structures to regulate its replication. These structures are involved in diverse processes including frameshifting during translation of the *pol* gene (32), regulation of transport of virus-specific RNAs by *rev* (39), and the activation of HIV-1 gene expression by *tat* (reviewed in references 20 and 33). One of the most widely studied of these HIV-1 RNA secondary structures is a highly conserved element known as TAR (41, 49). TAR, which comprises the first 57 nucleotides of the R region in the HIV-1 long terminal repeat (LTR), forms a stable RNA stem-loop structure (−24.8 kcal/mol [−104 kJ/mol]) that is essential for *tat* activation of HIV-1 gene expression (18, 41). Tat activation requires the preservation of the TAR RNA secondary structure in addition to the maintenance of a 3-nucleotide bulge and a 6-nucleotide loop element (reviewed in references 20 and 33). Viruses containing mutations that either disrupt the TAR RNA stem-loop structure, delete the bulge element, or alter the primary sequences of the loop exhibit marked decreases in gene expression and replication (24). Using a genetic selection with these TAR viral mutants, we recently isolated revertants that defined minimal TAR RNA sequences required for efficient HIV-1 gene expression (25). In the current study, we utilized these revertants in addition to a variety of other viruses containing mutations in TAR to demonstrate that TAR RNA is required for efficient reverse transcription and to define nucleotide sequences in TAR that are required for this process.

MATERIALS AND METHODS

Plasmid constructs. A plasmid (24) containing HIV-1 sequences extending from −160 to +988 was ligated with DNA fragments containing HIV-1 sequences extending from −20 to +80 obtained by PCR amplification of chromosomal DNA isolated from G418-resistant Jurkat cells infected with the TAR mutant viruses TAR1* and TAR2* (25). The TAR1* and TAR2* fragments

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were then ligated into the infectious construct pBRDH2-neo, and the neomycin resistance gene was inserted in place of the *nef* gene to allow G418 selection of cell lines containing viruses (24). The TAR mutant +15/+17 (19) was constructed as previously described and then ligated into the pBRDH2-neo construct. All constructs were confirmed by DNA sequencing (USB/Amersham). The runoff RNA used for quantitative reverse transcriptase was synthesized from a pGEM3Z construct that contained HIV-1 sequences from −22 to +242 with an internal deletion that extended from +80 to +140. The construct was linearized with the restriction enzyme *Xba*I, and in vitro-synthesized RNA was obtained by using SP6 RNA polymerase and gel purified.

Cell lines and virus stocks. To generate stable cell lines that produced the TAR1* and TAR2* mutants, the human embryonic kidney cell line 293 (22) was transfected with Lipofectamine (Gibco-BRL) by using 2 μg of the *Mro*I-linearized pBRDH2-neo construct containing the TAR1* or TAR2* mutation. Three days posttransfection the transfected 293 cells were serially diluted at 1:45, 1:15, and 1:5 in Iscove's medium supplemented with 5% newborn calf serum, 2.5% fetal bovine serum, 1% penicillin-streptomycin, and 1 mg of Geneticin (Gibco-BRL) per ml. The medium was changed every 4 to 5 days until cell foci grew to 2 mm in diameter. The cells were removed with cloning wells (Bellco Glass, Inc.), expanded, and assayed for capsid antigen (Ag) expression (p24 Ag) and reverse transcriptase activity in cell-free culture supernatants. The stable 293 cell lines that produced either the wild-type or TAR mutant viruses at nucleotide positions in TAR extending between +22 and +24, between +30 and +33, between +39 and +42, between +18 and +21 and also +39 and +42, or between +9 and +12 and also +39 and +42 have been previously described (24).

Jurkat cells were infected with 50 ng of p24 Ag for 2 h at 37°C; the cells were washed extensively to remove residual virus and then cultured in complete media. Four days postinfection the cells were cultured in media supplemented with 2 mg of Geneticin (G148) per ml. The G418-resistant Jurkat cells were obtained in approximately 2 weeks for the wild-type HIV-1 and 4 weeks for the HIV-1 TAR1* and TAR2* mutants, and these cells lines were stored in liquid nitrogen. Peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1-seronegative donors and activated as previously described (24).

HIV-1 wild-type or TAR mutant stocks were produced from newly confluent stable 293 cells that expressed each virus. RPMI containing 10% fetal bovine serum (heat inactivated), 1% penicillin-streptomycin, and 1% glutamine was added to each of the cell lines for 12 to 16 h at 37°C and 5% CO_2, and the medium was filtered by gravity flow through a 0.4-μm-pore-size membrane, separated into aliquots, and maintained at −80°C until used. Virus stocks produced in Jurkat cell lines stably infected with wild-type HIV-1 or TAR1* or TAR2* mutant virus were resuspended in fresh RPMI culture medium at 2×10^6 cells per ml and grown for 16 h, and the cells were pelleted by centrifugation at $400 \times g$ for 10 min. The culture supernatant was filtered as described elsewhere (59).

HIV-1 infection. For HIV-1 replication studies 2×10^6 Jurkat cells or activated PBMCs were incubated with wild-type, TAR1*, or TAR2* filtered virus stock for 2 h at 37°C and washed three times with culture medium to remove residual virus, and the cells were cultured in complete media and sampled for p24 Ag every 3 or 4 days. Experiments were performed three times with virus prepared from both stable 293 and Jurkat cell lines.

To perform single-cycle infections, virus stocks stored at −80°C for either wild-type or TAR mutant viruses were thawed at 37°C and the p24 Ag concentrations were normalized by using medium from uninfected cells that was filtered through a 0.4-μm-pore-size membrane. Virus supernatant containing approximately 75 ng of p24 Ag in a total volume of 7.5 to 10 ml of medium was adjusted to 5 mM $MgCl_2$ and 100 U (Kunitz) of DNase I (DP; Worthington Biochemical) and incubated at 30°C for 30 min. For heat activation, DNase I-treated virus stocks were treated at 60°C for 20 min. For each infection, 5×10^6 Jurkat cells or activated PBMCs were treated with DNase I-treated virus stocks (50 ng of p24 Ag) or heat-inactivated virus (25 ng of p24 Ag) and incubated at 37°C. Two hours postinfection, the cells were washed four times with 10 ml of complete culture medium and half of the cells were removed for analysis while the remainder continued for 24 h and then were harvested. Hirt lysis (27) was performed by washing the cell pellet in 1× phosphate-buffered saline, pelleting the cells at $400 \times g$ for 10 min, and gently resuspending the cells in 0.5 ml of Tris buffer (10 mM Tris [pH 7.0], 10 mM EDTA). The cell suspension was gently vortexed, lysis solution (10 mM Tris [pH 7.0], 10 mM EDTA, 1.2% sodium dodecyl sulfate [SDS], 2 mg of proteinase K per ml) was added, the mixture was incubated at 37°C for 2 h, 0.25 ml of 5.0 M NaCl was added, and the mixture was stored overnight on ice. The lysate was subjected to centrifugation at $16,000 \times g$ for 1 h, the supernatant was decanted, a phenol-chloroform-isoamyl alcohol (25:24:1) solution was added, and the mixture was vortexed and subjected to centrifugation at $14,000 \times g$ for 15 min. The aqueous layer was transferred to a fresh tube, and the nucleic acids were precipitated with 2 volumes of ethanol overnight at −20°C, pelleted by centrifugation at $16,000 \times g$ for 1 hour, and then resuspended in 50.0 μl of TE (10 mM Tris [pH 7.8], 0.1 mM EDTA).

HIV-1 internalization assay. The viral internalization assay was adapted from a previously described protocol (54). Briefly, either HeLa cells or HeLa $CD4^+$ cells were briefly treated with 1× trypsin (Gibco-BRL), and 2×10^5 cells of each type were infected in suspension with the same amount (100 ng of p24 Ag) of either wild-type or mutant virus. Each infection incubation lasted 2 h at 37°C; then the samples were washed four times with 1× phosphate-buffered saline to remove excess virus and cultured overnight. The cells were pelleted by centrifugation, and the cell pellet was suspended in 0.25 M Tris (pH 7.8) and lysed by repeated freezing-thawing (−80 and 37°C) and vortex treatment. The cell debris was pelleted by centrifugation at $16,000 \times g$ for 15 min, and the supernatant was assayed for p24 Ag by enzyme-linked immunosorbent assay (ELISA). These experiments were performed in triplicate with two independent virus stocks.

Reverse transcriptase assay and p24 Ag ELISA. Minor modifications were made to the mini-reverse transcriptase assay previously described (24). For each assay, 10 μl of cell-free culture supernatant was incubated with 5 μl of 10× RT buffer (500 mM Tris [pH 7.8], 75 mM KCl, 20 mM dithiothreitol, 50 mM MgCl, 0.5% Nonidet P-40) for 10 min at 37°C, followed by the addition of 35 μl of a reaction mix containing 250 ng of poly(rA) · oligo$(dT)_{12-18}$ (Pharmacia), 1.0 μCi of [^{35}S]thymidine 5′-(α-thio)triphosphate (NEG-038H; NEN Research Products), and 2 to 4 U of an RNase inhibitor (Boehringer Mannheim). After a 90-min incubation at 37°C, 5 μl of the reaction mixture was spotted onto a DEAE filter membrane (NA45; Schleicher and Schuell) and washed three times with 0.5 M sodium phosphate buffer (pH 6.8), and the incorporated counts were measured by liquid scintillation. Cell-free supernatants were assayed for the capsid antigen, p24, at a detection limit of 5 to 10 pg/ml by ELISA (DuPont-NEN) according to the manufacturer's instructions.

PCR analysis. Chromosomal DNA from Jurkat or 293 cells was isolated as previously described (24). Both the 5′ LTR and the 3′ LTR from Jurkat or 293 cells stably infected with the TAR mutant virus TAR1*, TAR2*, or +15/+17 were obtained by PCR. PCR reagents were obtained from Invitrogen, and *Taq* DNA polymerase was purchased from Gibco-BRL. Purified chromosomal DNA (200 ng) and 50 ng each of the oligonucleotide pair −436/−415 (5′-CCCAAA CAAGACAAGAGATTGA-3′, sense) plus +242/+219 (5′-CCTGCGTCGAGA GAGCTCCTCTGG-3′, antisense) (5′ LTR) or +8605/+8625 (5′-GCAGCTTT AGATATTAGCCAC-3′, sense) plus +9282/+9258 (5′-CTGCTAGAGATTTT TCCACACTGAC-3′, antisense) (3′ LTR) were subjected to PCR at 95, 55, and 72°C for 1 min at each temperature by using 1.25 U of *Taq* DNA polymerase. The expected 678- and 677-bp DNA products for the 5′ and 3′ LTRs, respectively, were ligated into the TA vector (Invitrogen) and analyzed by DNA sequence analysis. To normalize chromosomal DNA concentrations, a pair of oligonucleotide primers complementary to the first exon of the human β-globin gene between nucleotides 14 and 33 [5′-ACACAACTGTGTTCACTAGC-3′, sense) and between nucleotides 123 and 104 (5′-CAACTTCATCCACGT TCACC-3′, antisense) that produce a 110-bp band were used in 25 cycles of PCR at 93°C for 1 min and 65°C for 2 min. The samples were resuspended in 15 μl of loading dye (10 mM Tris [pH 8.0], 10 mM EDTA, 30% glycerol, 0.02% bromophenol blue, 0.02% xylene cyanol, and 0.02% orange G) and subjected to electrophoresis on a 6% polyacrylamide gel system at 250 V for 1 h (D600 gel solution; AT Biochem).

Nucleic acid concentrations of the Hirt lysates were normalized by "hot" PCR amplification (59) using the mitochondrial DNA-encoded cytochrome *c*-oxidase II (cyt-C-ox II) gene. Hirt lysates were serially diluted in 10-fold increments, and 5 μl of each dilution was assayed by PCR. *Taq* DNA polymerase was first inactivated with Taqstart Ab (Clontech) for 5 min at room temperature and then added to a master PCR mix that contained 1× optimized buffer D (Invitrogen), 0.1 mM deoxynucleoside triphosphates (dNTPs), 50 ng of an unlabeled oligonucleotide (5′-CACATGCAGCGCAAGTAGGT-3′, sense), and 25 ng of a ^{32}P-labeled oligonucleotide (5×10^8 cpm/μg) (5′-GGAAATGATTATGAGG GCGTG-3′, antisense) which hybridized to the human cyt-C-ox II gene. After a 95°C denaturation step for 5 min, 20 cycles of amplification were performed at 93°C for 1 min and 65°C for 2 min. The PCR products were separated by electrophoresis on a 5% polyacrylamide gel system. The dried gels were quantified on a Molecular Dynamics PhosphoImager and visualized by autoradiography. Generally, the cyt-C-ox II mitochondrial gene produced linear PCR amplification in 10^2 to 10^4 dilutions of the Hirt lysate.

Normalized Hirt lysate samples of exactly 5 μl were directly assayed for HIV-1 DNA by 30 to 32 cycles of hot PCR. Oligonucleotides detected the following size species of SF2 LTR DNA: (i) a 143-bp DNA fragment that contained R and U5 sequences between +42 and +62 (5′-GCTAACTAGGGAACCCACTGC-3′, sense) and between +182 and +158 (5′-CTGCTAGAGATTTTCCACACTG AC-3′, antisense), (ii) a 142-bp DNA fragment that contained R and U3 sequences between −49 and −30 (5′-TGGCGTGCCCTCAGATGCTG-3′, sense) and between +93 and +70 (5′-CCTCAATAAAGCTTGCCTTGAGTG-3′, antisense), and (iii) a 147-bp DNA fragment that contained the 5′-untranslated sequence and U5 between +242 and +219 (5′-CCTGCGTCGAGAGAGCTCCT CTGG-3′, antisense) and between +96 and +118 (5′-CAAGTAGTGTGTGCC CGTCTGTT-3′, sense).

Quantitative reverse transcriptase PCR was performed by using virion-associated RNA isolated from pelleted virus particles. DNase I-treated virus stocks for either the wild type or TAR mutant viruses were subjected to centrifugation at $16,000 \times g$ for 90 min and resuspended in 1× phosphate-buffered saline–1% bovine serum albumin (BSA). The viral suspensions were assayed for p24 Ag and reverse transcriptase activity. Equal amounts of p24 Ag (100 ng) were solubilized with Trisol (Gibco-BRL) according to the manufacturer's recommendations, and an in vitro-synthesized HIV-1 RNA was added to monitor RNA recovery and in vitro reverse transcription efficiency. Nucleic acids were precipitated overnight and recovered by centrifugation at $15,000 \times g$ at 2°C for 1 h. A visible pellet was washed with 70% ethanol, centrifuged, and resuspended in 30 μl of PCR TE.

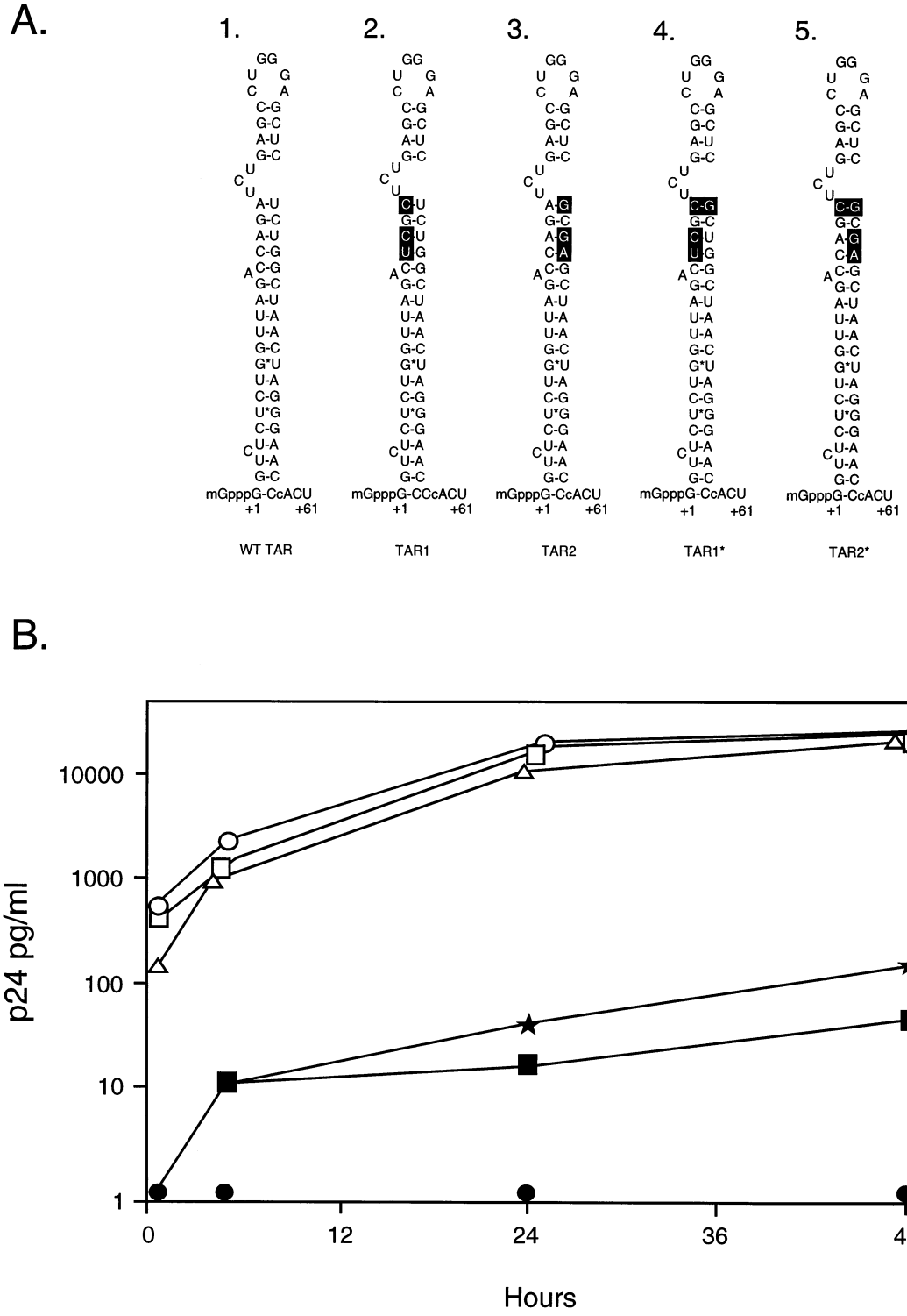

FIG. 1. Gene expression of HIV-1 TAR mutant viruses. (A) HIV-1 RNA from +1 to +61 is shown with the TAR RNA structures extending from +1 to +57 for wild-type (WT) TAR, the original TAR mutants TAR1 (+18/+21) and TAR2 (+39/+42), and the TAR revertants TAR1* [(+18/+21)/+39] and TAR2* [+21/(+39/+42]. The nucleotides in TAR that differ from those in the wild type are indicated. (B) G418-resistant Jurkat cell lines infected with either the wild-type virus (○), the revertant virus TAR1* (△) or TAR2* (□), or the original TAR RNA mutant virus TAR1 (★) or TAR2 (■), as well as uninfected Jurkat cells (●), were extensively washed, and cell-free supernatant was sampled for p24 Ag at 0, 4, 24, and 48 h. These results are representative of three independent experiments.

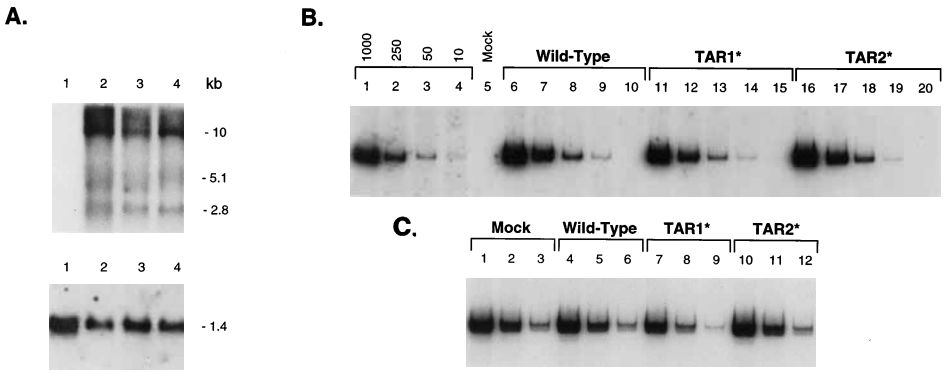

FIG. 2. Revertants in HIV-1 TAR produce wild-type levels of viral RNAs. (A) Northern analysis was performed with 10 μg of RNA isolated from either uninfected Jurkat cells (lane 1) or G418-resistant Jurkat cell lines infected with HIV-1 wild-type (lane 2), TAR1* (lane 3), or TAR2* (lane 4) virus. The Northern blot was probed with a ^{32}P-labeled DNA fragment containing HIV-1 nucleotides extending from 8050 to 8385 that can detect unspliced (10 kb), partially spliced (5.1 kb), or fully spliced HIV-1 RNA species (2.8 kb). The blot was also probed for a GADPH control RNA transcript (1.4 kb). (B) To determine the amount of provirus present in the G418-resistant Jurkat cells, quantitative PCR was performed on chromosomal DNA isolated from either uninfected Jurkat cells or Jurkat cells infected with HIV-1 wild-type, TAR1*, or TAR2* virus by using oligonucleotide primers that can amplify HIV-1 nucleotides extending from +91 to +242. The labeled products from 30 cycles of PCR are shown with molecular standards present at 1,000, 250, 50, and 10 copies (lanes 1 to 4, respectively); 100 pg of chromosomal DNA from uninfected Jurkat cells (lane 5); or 100, 20, 4, 0.8, or 0.16 pg of chromosomes isolated from Jurkat cells infected with HIV-1 wild type (lanes 6 to 10), TAR1* (lanes 11 to 15), or TAR2* (lanes 16 to 20). (C) The same chromosomal DNA was also analyzed by PCR using oligonucleotide primers complementary to the first intron of the human β-globin gene with 20, 4, or 0.8 pg of DNA isolated from uninfected Jurkat cells (lanes 1 to 3) or Jurkat cells infected with the wild type (lanes 4 to 6), TAR1* (lanes 7 to 9), or TAR2* (lanes 10 to 12).

Duplicate reaction mixtures that contained 5 μl of each viral RNA, 50 ng of the oligonucleotide primer (+242/+219) (5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′, antisense), and 5 μl of dimethyl sulfoxide were incubated at 65°C for 10 min. In vitro reverse transcription reactions were performed in the presence and absence of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL) with buffers provided by the manufacturer plus 0.1 mM dNTPs at 37°C for 30 min. Each reverse transcriptase reaction mixture was serially diluted in fivefold increments and assayed for the internal-control RNA by 30 cycles of hot PCR as previously described by using an oligonucleotide primer made to the polylinker region of the internal-control RNA (5′-CTTGCATGCCTGCAGGTCGACT-3′, forward) and HIV-1 sequences from +62 to +42 (5′-AAGCAGTGGGTTCCCTAGTTAG-3′, antisense). The serial dilutions were normalized to internal-control RNA concentrations and amplified by 30 cycles of hot PCR as described by using the HIV-1-specific oligonucleotide primer (+96/+118 [5′-CAAGTAGTGTGTGCCCGTCTGTT-3′, sense]) and a primer nested to the cDNA first-strand primer (+236/+214 [5′-CGAGAGAGCTCCTCTGGTTCTAC-3′, antisense]). The PCR products were then separated on a 5% polyacrylamide gel and quantified by Phosphorimager analysis (59).

Northern analysis. Total RNA extracted from Jurkat cells stably infected with HIV-1 wild type, TAR1*, or TAR2* were probed by Northern (RNA) analysis as previously described (24). Briefly, RNA was isolated from each cell line by using Trisol reagent (Gibco-BRL), and 10 μg of the RNA was separated on a 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane by using Turbo-blotter (Schleicher and Schuell). The baked filter (80°C, 2 h) was probed with a [^{32}P]dCTP-labeled, random-primed DNA fragment containing HIV-1 sequence +8050 to +8385 that hybridized to both spliced and unspliced HIV-1 RNA transcripts, and the blot was visualized by autoradiography.

Immunoblot analysis of HIV-1 virions. Virus stocks were pelleted by centrifugation at 15,000 × *g* for 90 min and resuspended in phosphate-buffered saline-BSA. The virion suspensions were assayed for both p24 Ag and reverse transcriptase activity as described elsewhere (24). An amount of virion suspension equivalent to 2 μg of p24 Ag was lysed in an equal volume of 2× Laemmli buffer, and 40 ng of p24 was subjected to electrophoresis on an SDS–15 or 7% polyacrylamide gel. The blot was transferred to nitrocellulose and probed with either a 1:5,000 dilution of purified human anti-HIV-1 immunoglobulin G (IgG) (ARRP 192; National Institutes of Health) or a 1:500 dilution of purified rabbit anti-gp120 polyclonal IgG, respectively. A second antibody, either horseradish peroxidase-conjugated rabbit anti-human or goat anti-rabbit, was diluted 1:2,000 and used for enhanced chemiluminescence detection (Amersham).

RESULTS

HIV-1 revertants in TAR have wild-type levels of gene expression. Previously, we reported a method to produce viruses that contain mutations in the HIV-1 TAR element (24). Because these viruses are markedly defective for transcriptional activation by Tat, they are not able to be isolated by conventional means. To obtain high titers of these viruses, the neo-

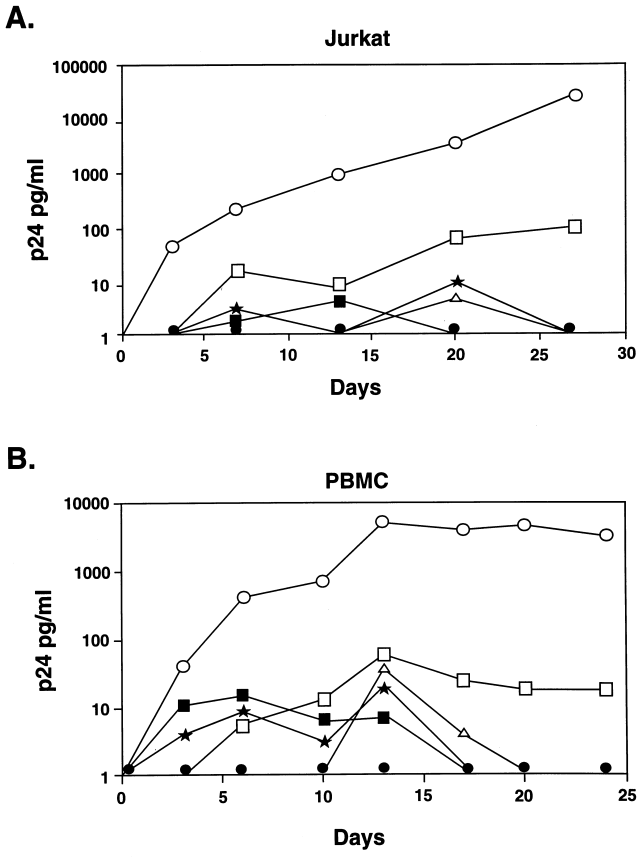

FIG. 3. Replication kinetics of HIV-1 wild-type and TAR mutant viruses. The replication kinetics of HIV-1 wild-type (○), TAR1* (△), TAR2* (□), TAR1 (★), and TAR2 (■) viruses were assayed in Jurkat cells (A) or activated PBMCs (B). Mock infections (●) were also assayed. For each infection, 5 × 10^6 cells were infected with supernatant containing 50 ng of p24 Ag for 2 h at 37°C, washed three times to remove excess virus, and resuspended in culture medium. Cell-free culture supernatant was sampled every 3 to 4 days and assayed for p24 Ag.

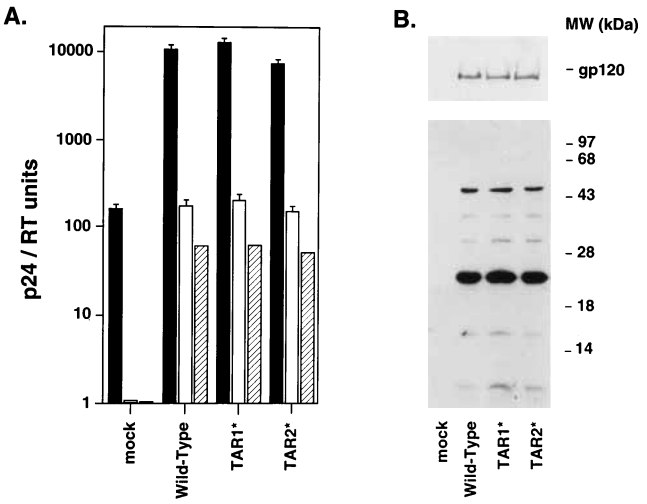

FIG. 4. Biochemical analysis of wild-type and TAR mutant virions. (A) Cell-free virus stocks (1 μg of p24 Ag) were isolated and assayed in triplicate for virion-associated reverse transcriptase activity by an ^{35}S-thymidine reverse transcriptase assay (■) and p24 Ag by ELISA (□). The standard deviations from the mean are indicated for each assay as well as the reverse transcriptase-to-p24 Ag ratio for each virion suspension (▨). (B) Western blot analysis was performed for a mock-infected sample (lane 1) or equal quantities of virions (40 ng of p24 Ag) prepared from the wild-type (lane 2), TAR1* (lane 3), and TAR2* (lane 4) viruses. HIV-1-specific proteins were detected by using either a purified rabbit anti-gp120 (IIIB) IgG (upper panel) or a purified anti-HIV-1 IgG (lower panel). The appropriate secondary conjugated antibody was detected by chemiluminescence. The mobilities of the molecular weight (MW) markers are indicated.

mycin resistance gene was inserted within the *nef* open reading frame and clonal 293 cell lines expressing each of these viruses were isolated. The presence of the adenovirus E1A and E1B proteins in 293 cells was able to complement the transcriptional defects of these TAR mutants and allow for the production of high-titer HIV-1 preparations (24, 35). Viruses containing mutations in the HIV-1 TAR RNA loop, bulge, or stem exhibited marked defects in replication following infection of Jurkat cells or PBMCs compared with wild-type HIV-1 produced under identical conditions (24). However, continuous culture of several Jurkat cell lines infected with the original TAR mutant viruses, designated TAR1 and TAR2 (Fig. 1A), spontaneously produced culture supernatant with wild-type levels of reverse transcriptase activity (25). This increased level of gene expression was due to compensatory mutations within TAR that restored the stem structure, resulting in TAR RNA structures designated TAR1* and TAR2* (Fig. 1A). The major effect of these compensatory mutations was to restore the TAR RNA bulge structure, permitting the binding of Tat and nearly wild-type activation of these TAR structures by Tat in transient expression assays (24).

To further investigate the replication of these TAR revertant viruses, we inserted the TAR elements of the revertants TAR1* and TAR2* into the parental HIV-1 construct pBRDH2-neo (24) and clonal 293 cell lines containing these viruses were isolated to ensure no additional mutations. Jurkat cells were infected with equal amounts of reverse transcriptase activity for either wild-type HIV-1, viruses containing the original TAR mutants TAR1 and TAR2, or the revertants TAR1* and TAR2*. These cells were placed under drug selection with G418. G418-resistant Jurkat cell lines infected with wild-type virus were obtained at 2 weeks postinfection, while TAR1* and TAR2* viruses required 4 weeks and TAR1 and TAR2 viruses required 6 weeks (24). The rate at which populations of Jurkat cells became G418 resistant was reproducible in three separate experiments. PCR amplification of chromosomal DNA and subsequent DNA sequencing confirmed that no additional LTR mutations were present in these viruses (data not shown). These results suggested that the TAR1* and TAR2* mutant viruses which were previously demonstrated to give nearly wild-type levels of Tat-induced gene expression (25) may have an additional defect in the virus life cycle which occurred prior to integration.

To compare the level of HIV-1 gene expression in these G418-resistant Jurkat cell lines, equal quantities of these cells were cultured and cell-free supernatants were assayed at 0, 4, 24, and 48 h for capsid antigen (p24 Ag). Jurkat cells containing either the wild type or the TAR1* and TAR2* revertants produced approximately 10 to 20 ng of p24 Ag per ml at 48 h postinoculation, while Jurkat cells harboring the original TAR mutant viruses TAR1 and TAR2 expressed approximately 150-

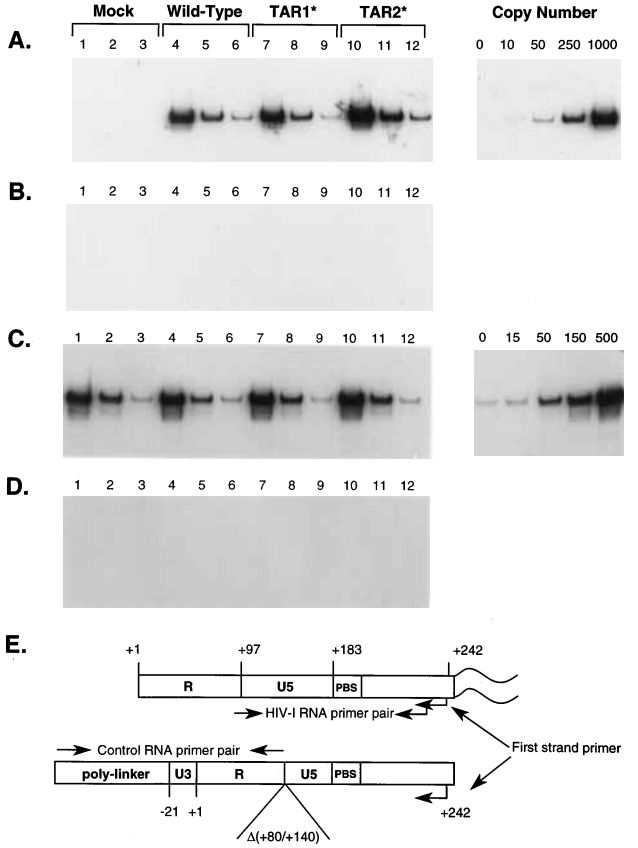

FIG. 5. Analysis of HIV-1-specific RNA isolated from partially purified wild-type or TAR revertant viruses. Virion-associated RNA (100 ng of p24 Ag) was isolated from wild-type-, TAR1*-, TAR2*-, or mock-infected samples, and then 5×10^{-4} μg of an in vitro-synthesized internal control runoff RNA was added to each sample. Both virion RNA (A and B) and internal-control RNA (C and D) were reverse transcribed in the presence (A and C) or absence (B and D) of M-MLV reverse transcriptase by using an oligonucleotide primer, +242 to +219, that annealed to both HIV-1 RNA and internal-control RNA. Nested PCR (E) was performed on fivefold serially diluted reverse transcription reaction mixtures for mock (lanes 1 to 3), wild-type (lanes 4 to 6), TAR1* (lanes 7 to 9), and TAR2* (lanes 10 to 12) infections by using oligonucleotide primers capable of distinguishing HIV-1 cDNA from the internal-control cDNA. Molecular standards equal to 1,000, 250, 50, and 10 copies for HIV-1 DNA or the internal-control plasmid present at 500, 150, 50, and 15 copies are shown on the right. The labeled DNA species were quantified on a Molecular Dynamics PhosphoImager. These results are representative of three identical experiments and two independent virus stocks.

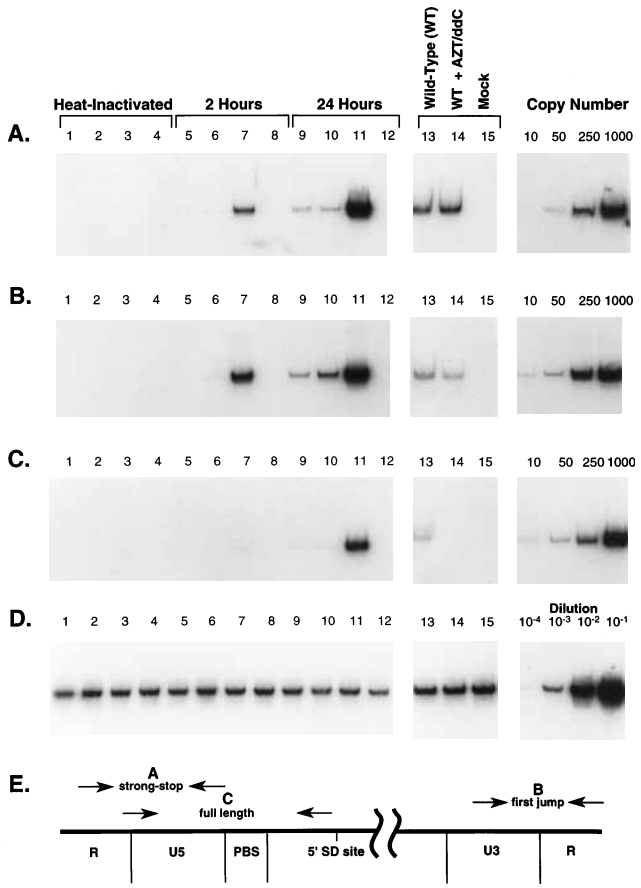

FIG. 6. Analysis of reverse transcription of wild-type HIV-1 and TAR revertants following single-cycle infections of PBMCs. DNase I-treated virion preparations (50 ng of p24 Ag) for TAR1* (lanes 1, 5, and 9), TAR2* (lanes 2, 6, and 10), wild-type HIV-1 (lanes 3, 7, and 11), or mock (lanes 4, 8, and 12) treatment were used to infect 5×10^6 activated PBMCs. Identical infections were prepared with the same virus stocks that were heat inactivated at 60°C for 20 min. Cytoplasmic DNA prepared at 2 and 24 h postinfection were assayed by quantitative PCR using primers specific for strong-stop DNA (+42/+62 and +182/+158) (A), the first-strand jump (−49/−30 and +93/+70) (B), or full-length viral DNA (+96/+118 and +242/+219) (C). The molecular standards present at 1,000, 250, 50, and 10 copies are indicated for each primer pair (A through C, lanes 13 to 15). AZT, zidovudine; ddC, dideoxycytosine. (D) The concentration of the recovered nucleic acids for each of the samples was determined by using primers that could detect the mitochondrial gene cyt C-ox II (lanes 1 to 12). A serially diluted Hirt lysate standard (10^{-4} to 10^{-1}) was assayed in each PCR analysis. All PCRs were performed within the linear range of each PCR amplification assay. This experiment was performed four times with two independent virus stocks. (E) Positions of the primers used in PCR analysis and positions of the U3, R, and U5 elements and the 5′ splice donor (SD) site.

to 625-fold-lower quantities of p24 Ag (Fig. 1B). This experiment, which was representative of three similar experiments, demonstrated that the level of HIV-1 gene expression for the TAR revertant viruses was not significantly reduced compared with the wild-type HIV-1. Northern blot analysis of G418-resistant Jurkat cell lines expressing the original TAR1 and TAR2 mutant viruses previously demonstrated greater than 20-fold decreases in the levels of HIV-1 RNAs compared with wild-type virus (24). However, the steady-state RNA levels of the 10-, 5.1-, and 2.8-kb HIV-1 transcripts isolated from Jurkat cells infected with the wild-type, TAR1*, and TAR2* viruses did not markedly differ (Fig. 2A, lanes 2 to 4). It was possible that the high level of gene expression in the G418-resistant Jurkat cell lines was due to the integration of multiple copies of HIV-1 provirus containing TAR1* and TAR2*. Chromosomal DNA was prepared from uninfected cells (Fig. 2B, lane 5) or G418-resistant Jurkat cells infected with either the wild type (Fig. 2B, lanes 6 to 10), TAR1* (Fig. 2B, lanes 11 to 15), or TAR2* (Fig. 2B, lanes 16 to 20), and the amount of proviral DNA was analyzed with HIV-1-specific primers by using 25 cycles of hot PCR as described in Materials and Methods. This analysis indicated that no substantial differences in the relative copy number of HIV-1 were present in these Jurkat cell lines. No differences in the relative concentrations of chromosomal DNA isolated from mock-, wild-type-, TAR1*-, and TAR2*-infected Jurkat cells were noted with primers specific for a β-globin control gene (Fig. C, lanes 1 to 12). These results indicate that the similar levels of gene expression in the G418-resistant Jurkat cells were not due to differences in the amounts of integrated provirus.

HIV-1 revertants in TAR exhibit marked defects in replication. The replication kinetics of the revertants, TAR1* and TAR2*, and the original TAR mutants, TAR1 and TAR2, were then compared with that of wild-type HIV-1 following infection of either Jurkat cells or PBMCs. Following infection of each cell type with similar amounts of p24 Ag, the cell-free supernatant was sampled every 3 to 4 days and assayed for p24 Ag. Jurkat cells (Fig. 3A) and peripheral blood lymphocytes (Fig. 3B) infected with wild-type virus had no detectable p24 Ag following infection, but the level of p24 Ag markedly increased to maximal levels between 12 to 28 days postinfection. In contrast, the original TAR mutants TAR1 and TAR2 gave extremely low levels of p24 Ag which did not significantly increase following 28 days of infection of either Jurkat cells or PBMCs (Fig. 3). Surprisingly, both the TAR1* and the TAR2* viruses also replicated poorly in both Jurkat cells and PBMCs, exhibiting defects in replication ranging from 100- to 2,000-fold compared with the wild-type virus (Fig. 3). These experiments, which were repeated with virus stocks prepared from G418-resistant Jurkat and 293 cell lines, indicated that the replication defects were not due to differences in the cell lines from which the viruses were prepared (data not shown). Thus, even though the TAR revertants gave high levels of HIV-1 gene expression, they exhibited a dramatic decrease in viral replication, suggesting an additional role for TAR in the virus life cycle.

HIV-1 revertants in TAR do not exhibit changes in protein or RNA content. To determine if the decreased infectivity of the TAR1* and TAR2* viruses resulted from defects in viral structure, cell-free viral supernatants were analyzed for both virion-associated reverse transcriptase enzymatic activity and p24 Ag. No significant variations were evident between the wild type and the TAR1* or TAR2* revertants (Fig. 4A). Western blot analysis was then performed on the cell-free viruses isolated from the G418-resistant Jurkat cell lines by using purified human anti-HIV-1 IgG (Fig. 4B, lower panel) or purified rabbit anti-gp120 (IIIb) IgG (Fig. 4B, upper panel). Equal quantities of proteins with approximate mobilities of 66/51, 41, 31, 24, 17, and 10 kDa were detected for the wild-type, TAR1*, and TAR2* virions (Fig. 4B, lower panel). Western blot analysis using the purified rabbit anti-gp120 also demonstrated similar quantities of gp120 for each of the virion preparations (Fig. 4B, upper panel). Thus, the decreased replication of the TAR1* and TAR2* mutants compared with wild-type virus could not be attributed to differences in the reverse transcriptase activity or in the proportion of viral structural proteins.

We investigated whether the defect in viral replication of the TAR revertants was due to decreased levels of virion-associated RNA. Viruses isolated from G418-resistant Jurkat cells infected with either the wild type, TAR1*, or TAR2* were

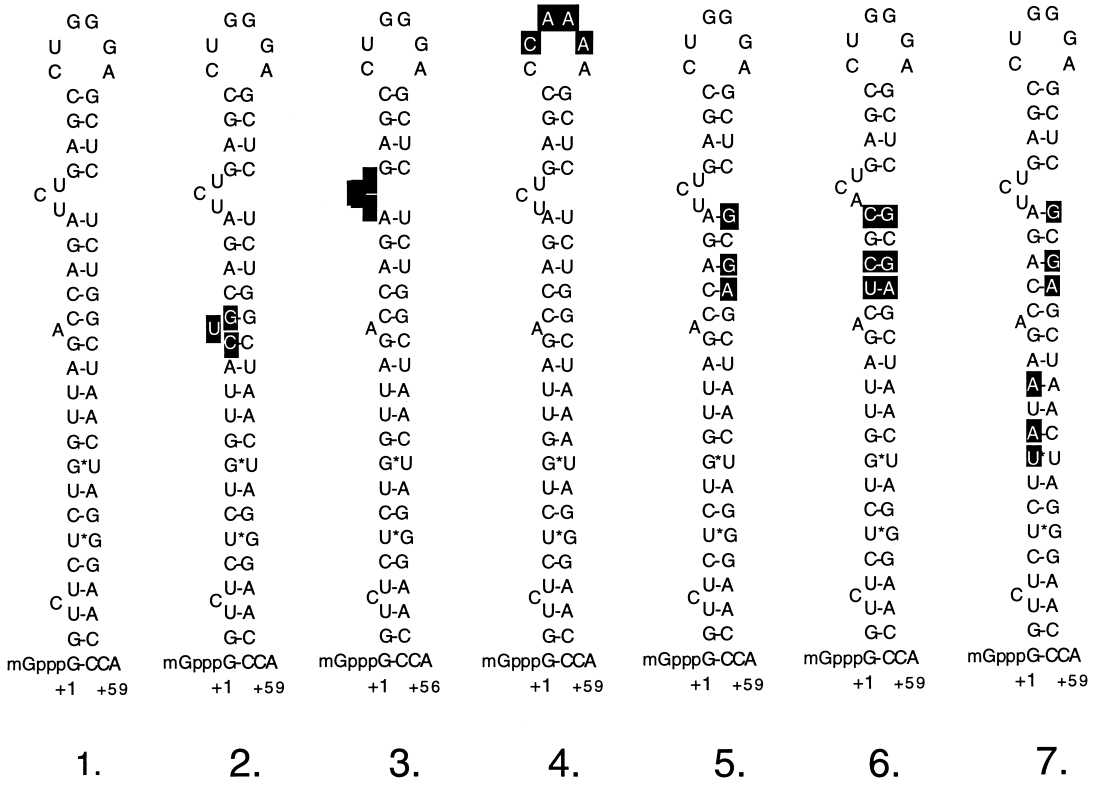

FIG. 7. Schematic of different HIV-1 LTR TAR mutations. A portion of the HIV-1 LTR extending from +1 to +59 is shown for the wild type and the TAR mutant viruses +15/+17, +22/+24, +31/+34, +39/+42, +18/+21–+40/+43, and +9/+12–+40/+43 (diagrams 1 to 7, respectively).

pelleted and assayed for virion-associated reverse transcriptase activity and p24 Ag. The RNA isolated from equal amounts of each of the viral preparations was reverse transcribed by using a first-strand oligonucleotide primer in the presence or absence of M-MLV reverse transcriptase. The reactions were serially diluted fivefold and assayed for HIV-1 virion cDNA by using PCR with primer pairs capable of discriminating an internal-control RNA from the virion-associated RNA (Fig. 5E). PCR amplification of the cDNAs corresponding to either the wild-type, TAR1*, or TAR2* virus gave similar levels of labeled PCR products in corresponding dilutions (Fig. 5A, lanes 4 to 12). Likewise, the internal-control cDNA produced a labeled PCR product of similar intensity for all viruses, indicating that both the RNA recovery and reverse transcriptase efficiencies were similar for each viral RNA (Fig. 5C, lanes 4 to 12). The mock-infected supernatant analyzed with M-MLV reverse transcriptase (Fig. 5A, lanes 1 to 3) or the reactions lacking M-MLV reverse transcriptase yielded no labeled PCR products (Fig. 5B and D, lanes 1 to 12). Thus, we concluded that the defects in viral replication with TAR1* and TAR2* were not due to altered packaging of viral RNA (3, 10, 26).

HIV-1 revertants in TAR exhibit defects in reverse transcription. We next investigated the mechanism for the replication defects seen with TAR1* and TAR2* viruses. First, the relative infectivities of the wild-type and TAR revertant viruses were compared in a viral internalization assay. Either HeLa $CD4^+$ cells or HeLa cells were infected for 2 h with 100 ng of p24 Ag of each virus stock followed by extensive washing. The non-specifically bound p24 Ag detected in HeLa cell lysates was subtracted from the quantity of p24 Ag in lysates prepared from the virus-infected HeLa $CD4^+$ cells. Approximately equal quantities of p24 Ag were present in the HeLa $CD4^+$ lysates infected with the wild type, TAR1*, and TAR2*, indicating that reduced binding or internalization of the revertant viruses was not responsible for defective viral replication (data not shown).

We next assayed the abilities of the wild type and TAR revertants to undergo reverse transcription. During reverse transcription, DNA synthesis is initiated at the PBS site, continues through the U5 region of the LTR, and terminates at the end of the 5′ repeat (5′ R) (21, 53, 58). This DNA, referred to as minus-strand strong-stop DNA, then "jumps" to the 3′ R element which serves as the primer for DNA synthesis through the U3 region (28, 29). Several HIV-1 DNA intermediates are synthesized prior to the generation of a full-length, double-stranded DNA molecule that contains two complete LTRs (U3-R-U5) (53). Single-cycle infections were performed with wild-type and TAR mutant viruses, and total cytoplasmic nucleic acids were assayed for the presence of HIV-1 DNA intermediates. Activated PBMCs were infected with DNase I-treated viral supernatants obtained from the G418-resistant Jurkat cell lines producing TAR1*, TAR2*, and wild-type virus. Nuclease treatment of the cell-free viral supernatants was performed to remove contaminating chromosomes and nuclei from the HIV-1-producing cells. Following extensive washing to remove excess virus, nucleic acids were recovered by Hirt lysis at either 2 h or 24 h postinfection, and PCR analysis was performed to characterize the DNA structures which were produced. HIV-1-specific oligonucleotide primer pairs were used in the PCR analysis that detected either a 141-bp DNA fragment corresponding to minus-strand strong-stop DNA, a 123-bp DNA fragment corresponding to DNA synthesized after the first-strand jump, or a 146-bp DNA fragment representing full-length HIV-1 DNA as illustrated in Fig. 6E. The

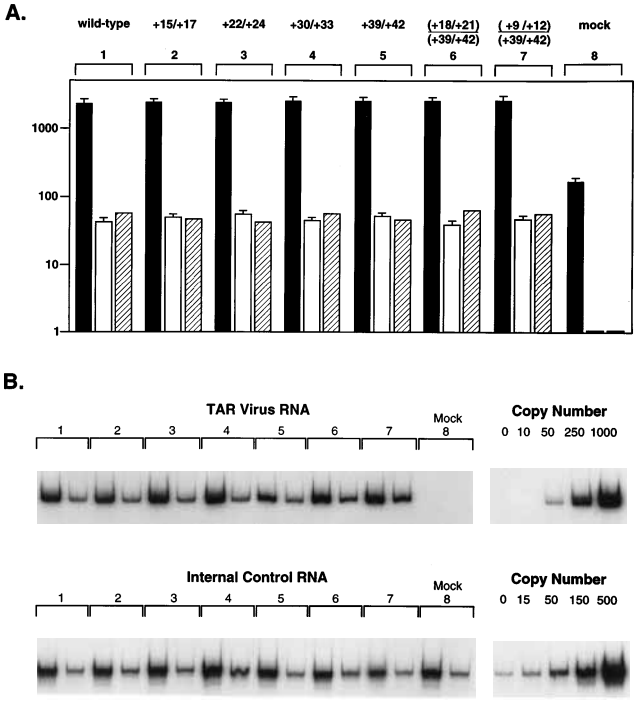

FIG. 8. Biochemical analysis of wild-type and TAR mutant viruses. (A) Wild-type HIV-1 and TAR mutant viruses +15/+17, +22/+24, +31/+34, +39/+42, +18/+21–+40/+43, and +9/+12–+40/+43 were produced in clonal 293 cell lines containing each of these viruses. DNase I-treated cell-free virus stocks (200 ng of p24 Ag) were pelleted and assayed in triplicate for virion-associated reverse transcriptase activity (solid bars) and for p24 Ag (open bars). The standard deviations from the means are indicated. Hatched bars, reverse transcriptase-to-p24 Ag ratio. (B) Quantitative reverse transcriptase PCR analysis of virion RNAs from wild-type and TAR mutant viruses was performed. Virion-associated RNA and an in vitro-synthesized internal-control RNA were isolated from DNase I-treated viral supernatants (100 ng of p24 Ag) for the wild type (lane 1) or the TAR mutant virus stocks +15/+17 (lane 2), +22/+24 (lane 3), +31/+34 (lane 4), +40/+43 (lane 5), +18/+21–+40/+43 (lane 6), and +9/+12–+40/+43 (lane 7). Both virion RNA (upper panel) and the internal-control RNA (lower panel) were hybridized to a first-strand oligonucleotide primer that encompassed HIV-1 sequences from +242 to +219 and reverse transcribed in the presence or absence (not shown) of M-MLV reverse transcriptase. Nested PCR was performed on fivefold serially diluted cDNA and detected by quantitative PCR using both a high dilution and a low dilution for each sample. Molecular standards present at the indicated copy number verified that all PCRs were performed in the linear range of the assays. Quantification of the labeled DNA species was performed on a Molecular Dynamics PhosphoImager. This experiment is representative of three independent experiments performed on two separately prepared virus stocks.

relative nucleic acid concentrations of each sample were normalized by PCR quantitation of the mitochondrion-encoded cyt-C-ox II gene.

At 2 h postinfection, wild-type HIV-1 produced strong-stop DNA (Fig. 6A, lane 7) that increased sevenfold by 24 h postinfection (Fig. 6A, lane 11). In contrast, PBMCs infected with HIV-1 TAR1* or TAR2* exhibited 23- and 18-fold decreases, respectively, in the amount of strong-stop DNA (Fig. 6A, lanes 5, 6, 9, and 10) compared with cells infected with the wild-type virus (Fig. 6A, lanes 7 and 11). Likewise, PCR analysis of TAR1* and TAR2* DNAs either synthesized after the first-strand jump (Fig. 6B, lanes 5, 6, 9, and 10) or fully synthesized (Fig. 6C, lanes 5, 6, 9, and 10) indicated approximately 20-fold decreases in their levels relative to that seen with wild-type virus at both 2 and 24 h postinfection (Fig. 6B and C, lanes 7 and 11). PBMCs treated with the reverse transcriptase inhibitors zidovudine and dideoxycytosine demonstrated that, although minus-strand strong-stop DNA was synthesized (Fig. 6A, lane 14), full-length DNA synthesis was inhibited (Fig. 6C, lanes 13 to 15). No labeled PCR products were detected in the mock-infected lysates (Fig. 6A through C, lane 12) nor in lysates prepared from cells infected with heat-inactivated virus (Fig. 6A through C, lanes 1 to 4). These data demonstrated that TAR1* and TAR2* viruses displayed decreased reverse transcription kinetics and that a block occurred prior to completion of minus-strand strong-stop DNA synthesis. However, this block did not interfere either with the first-strand jump or with the synthesis of full-length HIV-1 DNA, since approximately proportional levels of DNA product were observed for both the TAR mutant and the wild-type virus. Analysis with a second primer pair that detected sequences within the U5 regions of these viruses gave similar results (data not shown), thus confirming that viruses containing TAR mutations exhibited alterations in the early steps of reverse transcription.

Preservation of TAR RNA secondary structure and loop sequences is required for efficient reverse transcription. We then mapped the elements within TAR that were critical for efficient HIV-1 reverse transcription. Supernatants containing wild-type and TAR mutant viruses were prepared from stable, G418-resistant 293 cell lines (24). Viruses were produced that contained TAR mutations that disrupted the TAR RNA stem at nucleotides +15 to +17, deleted the TAR RNA bulge from nucleotide +22 to +24, altered TAR RNA loop sequences from +30 to +33, disrupted the upper TAR RNA stem between nucleotides +39 and +42, mutated nucleotides between +18 and +21 and between +39 and +42 but maintained a stable TAR RNA stem structure, and disrupted the TAR RNA stem structure between +9 and +12 and between +39 and +42. A diagram of the different TAR mutants used in this analysis is shown in Fig. 7.

Equal quantities of the above viruses were obtained by centrifugation of viral supernatants which were then assayed for both reverse transcriptase activity and p24 Ag (Fig. 8A). The reverse transcriptase/p24 Ag ratios were within the range observed for the wild-type virus (Fig. 8A). Western blot (immunoblot) analysis of the virion suspensions using both a human anti-HIV-1 IgG and a rabbit anti-gp120 (IIIb) IgG indicated similar levels of proteins for the HIV-1 wild-type and TAR mutant viruses (data not shown). Intravirion RNA was isolated from equal quantities of each of the pelleted virions and incubated in the presence or absence (not shown) of M-MLV reverse transcriptase along with a first-strand primer and an internal-control RNA. The samples were serially diluted in fivefold increments and assayed by PCR. A labeled PCR product of similar intensities in the corresponding dilutions for the wild-type (Fig. 8B, upper panel, lane 1) and each of the TAR mutants (Fig. 8B, upper panel, lanes 2 to 7) was detected. Comparable levels of cDNA synthesized from the internal-control RNA were detected in all reactions performed in the presence of M-MLV reverse transcriptase (Fig. 8B). No labeled DNA species were observed for the reverse transcriptase reaction performed with the mock sample (Fig. 8B, upper panel, lane 8) or in absence of M-MLV reverse transcriptase (data not shown). Thus, the TAR nucleotide substitutions or deletions did not alter the encapsidation of genomic RNA into the virion.

Activated PBMCs were infected with equal quantities of these DNase I-treated viral supernatants. Nucleic acids were isolated from the cytoplasm at 2 and 24 h postinfection and then assayed by PCR for the presence of HIV-1 minus-strand strong-stop DNA (Fig. 9A), first-strand jump (Fig. 9B), or full-length DNA (Fig. 9C). A labeled PCR product representative of minus-strand strong-stop DNA was present in nucleic

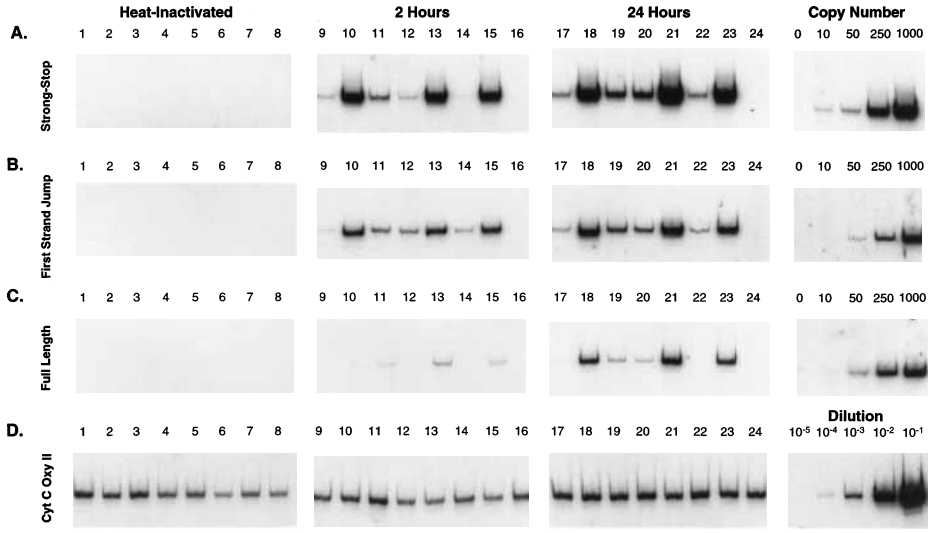

FIG. 9. Analysis of reverse transcription following single-cycle infections of PBMCs with HIV-1 wild-type or HIV-1 TAR mutant viruses. DNase I-treated supernatants (50 ng of p24 Ag) for either the wild-type HIV-1 (lanes 7, 15, and 23) or the TAR mutant viruses +15/+17 (lanes 1, 9, and 17), +22/+24 (lanes 2, 10, and 18), +31/+34 (lanes 3, 11, and 19), +40/+43 (lanes 4, 12, and 20), +18/+21–+39/+42 (lanes 5, 13, and 21, and +9/+12–+40/+43 (lanes 6, 14, and 22) were used to infect 5×10^6 activated PBMCs. Lanes 1 to 7, heat-inactivated supernatants; lanes 8, 16, and 24, mock supernatants. Cytoplasmic DNA was prepared from the infected cells at 2 h (lanes 9 to 16) and 24 h (lanes 17 to 24) postinfection and assayed as previously described by quantitative PCR using primers specific for strong-stop DNA (A), the first-strand jump (B), or the full-length viral DNA (C). Molecular standards which are present at 1,000, 250, 50, and 10 copies are shown on the right. (D) Total nucleic acids were normalized to mitochondrial DNA content by using primers that specifically amplify the cyt C-ox II gene (lanes 1 to 24). A 10-fold serially diluted Hirt lysate standard (10^{-5} to 10^{-1}) indicated that amplification was in the linear range of the assay. These experiments were performed at least three times with two independent virus stocks.

acids isolated from cells infected with wild-type virus at 2 h, and the amount of this species increased fivefold at 24 h postinfection (Fig. 9A, lane 15 versus lane 23). The amount of minus-strand strong-stop DNA at either 2 h or 24 h postinfection was reduced approximately 10- to 30-fold relative to that of wild-type virus for the TAR stem disruption mutants +15/+17 (Fig. 9A, lanes 9 and 17), +39/+42 (Fig. 9A, lanes 12 and 20), and +9/+12–+39/+42 (Fig. 9A, lanes 14 and 22), as well as the loop mutant +30/+33 (Fig. 9A, lanes 11 and 19). However, mutations that deleted the TAR RNA bulge sequences (+22/+24) (Fig. 9A, lanes 10 and 18) or that altered the primary sequence of the TAR RNA stem but maintained stem base pairing (+18/+21/+39/+42) (Fig. 9A, lanes 13 and 21) had levels of strong-stop DNA similar to that seen with the wild type. This same pattern of reverse transcription products was also observed for the different TAR mutants by using primers to detect DNA synthesized after the first-strand jump (Fig. 9B, lanes 9 to 15 and lanes 17 to 23) or full-length viral DNA (Fig. 9C, lanes 9 to 15 and lanes 17 to 23). The fold reduction for the TAR mutants relative to wild-type virus in the synthesis of the first-jump DNA was slightly less (8- to 20-fold) than that seen with the strong-stop or full-length DNA. No labeled PCR products were detected in the mock-infected lysates (Fig. 9A through C, lanes 8, 16, and 24) nor in lysates prepared from cells infected with heat-treated viral supernatants (Fig. 9A to C, lanes 1 to 7). Analysis with a second primer pair that detected sequences within the U5 region also gave similar results (data not shown). This analysis indicated that early steps of HIV-1 reverse transcription were markedly altered for viruses containing mutations that disrupted the TAR RNA stem structure or altered the TAR RNA loop sequences, while the bulge sequences seemed to be dispensable.

DISCUSSION

Reverse transcription of HIV-1 requires a $tRNA_3^{Lys}$ molecule to hybridize to the RNA PBS and the combined activities of the RNA-dependent DNA polymerase and RNase H. During reverse transcription, the U5 and 5′ R genomic RNA sequences are converted into a DNA copy called the minus-strand strong-stop DNA (5′ U5-R 3′) which is liberated by RNase H and then hybridizes to the 3′ R genomic RNA sequences during the first-strand jump to allow continued DNA synthesis (28, 29, 53, 58). In the current study, we demonstrate that the process of HIV-1 reverse transcription requires more complex regulation than previously described. An additional regulatory element, TAR RNA, is critical for the synthesis of strong-stop DNA during reverse transcription in addition to its well-described role in regulating HIV-1 gene expression in response to the transactivator Tat (20, 33).

Subtle point mutations in TAR, such as those present in the TAR1* and TAR2* viruses that either do not alter or only slightly decrease (less than twofold) viral gene expression, result in viruses which synthesize greatly diminished levels of minus-strand strong-stop DNA (15- to 30-fold). This defect in minus-strand strong-stop DNA is reflected in marked decreases in full-length DNA synthesis and subsequent decreases in viral replication. Analyses of a number of different TAR mutant viruses indicate that disruption of both the TAR RNA stem secondary structure as well as the primary sequence of the TAR RNA loop decreases DNA synthesis initiated from the HIV-1 PBS. However, two other viruses containing TAR RNA mutations which either delete the TAR RNA bulge (Δ+22/+24) or alter the primary sequence of TAR RNA but maintain stem secondary structure (+18/+21/+39/+42) have wild-type levels of strong-stop DNA. The TAR mutations that were analyzed did not markedly alter the process in which the 5′ TAR cDNA is translocated to the complementary 3′ TAR element nor alter the amount of RNA dimerization or encapsidation (7, 8, 11, 26). These results indicate that the strong conservation of TAR sequences in different HIV-1 isolates and the rapid rate of reversion seen with HIV-1 mutants that disrupt the lower portion of the TAR RNA stem (34) may be due

to the critical role of TAR not only in transcriptional activation (34) but also in regulating reverse transcription.

It is somewhat surprising that a stable TAR RNA secondary structure functions to increase rather than decrease the ability of reverse transcriptase to synthesize minus-strand strong-stop DNA. However, in vivo and in vitro studies with other retroviruses including Rous sarcoma virus (12, 13) and M-MLV (42) have identified RNA secondary structures downstream of the PBS that regulate the initiation of reverse transcription. Rous sarcoma virus contains a U5 RNA secondary structure which flanks the PBS and regulates the initiation of reverse transcription in an orientation-dependent manner (1, 2, 12, 13). Mutations within the Rous sarcoma virus U5 RNA stem-loop spacer virtually eliminate the initiation of reverse transcription. In vitro assays of HIV-1 reverse transcription indicate that deletions of the 5′ R element decrease the initiation of reverse transcription (4). Furthermore, HIV-1 mutants carrying mutations in the PBS replicate with delayed kinetics and revert rapidly to wild-type sequences (15, 37, 38, 47, 55, 56). These observations support a model in which the initiation of HIV-1 reverse transcription requires a defined complex consisting of at least $tRNA_3^{Lys}$ and the PBS, the p66/p51 reverse transcriptase heterodimer, and TAR RNA. These elements may be necessary to promote a structurally favored initiation complex required for efficient reverse transcription (31). The mechanism by which TAR RNA may stabilize this complex is not known but may involve the interaction with specific viral or cellular proteins or may be mediated by the structure of TAR RNA.

Viral proteins other than reverse transcriptase are involved in the control of HIV-1 reverse transcription. For example, the viral nucleocapsid protein NCp7 is tightly associated with the viral RNA and can facilitate RNA dimerization and encapsidation, tRNA packaging and annealing at the PBS (6, 46), and strand transfer in cooperation with reverse transcriptase (45, 46). Since the initiation of reverse transcription likely occurs within the viral capsid, NCp7 may play a generalized role in stabilization, maturation, and perhaps the activation of a ribonucleoprotein complex in which TAR RNA is an important component. HIV-1 "accessory" proteins including nef (51), vif (54), and integrase (40) have also been reported to affect reverse transcription. However, the reduced levels of DNA synthesis seen with these mutants may be the result of defective viral morphology (14, 17) or defects in the viral uncoating process. Since TAR RNA is critical for Tat function, the question arises whether the HIV-1 Tat protein may be involved in the control of reverse transcription. HIV-1 mutants with mutations in the *tat* gene whose transcriptional defect is complemented with other viral transactivators still exhibit marked defects in replication (30). This suggests that Tat likely functions in other processes in the HIV-1 life cycle exclusive of transcriptional activation. Tat binds to the TAR RNA bulge sequences between +22 and +24 to facilitate increases in HIV-1 gene expression (9, 16, 50, 57), though an HIV-1 mutant (this study) with a deletion of the TAR RNA bulge sequences does not exhibit defects in reverse transcription. Thus, if Tat is involved in the ability of TAR RNA to increase reverse transcription, a different TAR RNA recognition element other than the bulge and/or viral and cellular factors that could stabilize Tat interactions with the reverse transcription initiation complex would likely be involved.

While reverse transcription occurs rapidly in recently infected T-lymphocyte lines such as Jurkat, H9, or stimulated PBMCs, quiescent PBMCs can initiate but not complete reverse transcription. However, stimulation of these quiescent cells with mitogens initiates reverse transcription (59, 60). Several possibilities could explain this observation: the release of sequestered intracellular nucleotides (43), the uncoating of the viral core, or the modification of cellular or viral regulators of reverse transcription. Whether the block in reverse transcription in quiescent PBMCs could be due to the modification of viral or cellular factors that interact with TAR RNA remains to be determined. In summary, we have used a genetic approach to demonstrate that a highly conserved HIV-1 transcriptional control element, TAR RNA, regulates two important steps in the viral life cycle. The ability to identify additional regulatory elements involved in the control of the reverse transcription complex should increase our ability to identify additional steps in this process that are possible targets for antiviral strategies to inhibit HIV-1 replication.

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