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Differential Growth Kinetics Are Exhibited by Human Immunodeficiency Virus Type 1 TAR Mutants

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The human immunodeficiency virus type 1 (HIV-1) TAR element is critical for the activation of gene expression by the transactivator protein, Tat. Mutagenesis has demonstrated that a stable stem-loop RNA structure containing both loop and bulge structures transcribed from TAR is the major target for *tat* activation. Though transient assays have defined elements critical for TAR function, no studies have yet determined the role of TAR in viral replication because of the inability to generate viral stocks containing mutations in TAR. In the current study, we developed a strategy which enabled us to generate stable 293 cell lines which were capable of producing high titers of different viruses containing TAR mutations. Viruses generated from these cell lines were used to infect both T-lymphocyte cell lines and peripheral blood mononuclear cells. Viruses containing TAR mutations in either the upper stem, the bulge, or the loop exhibited dramatically decreased HIV-1 gene expression and replication in all cell lines tested. However, we were able to isolate lymphoid cell lines which stably expressed gene products from each of these TAR mutant viruses. Though the amounts of virus in these cell lines were roughly equivalent, cells containing TAR mutant viruses were extremely defective for gene expression compared with cell lines containing wild-type virus. The magnitude of this decrease in viral gene expression was much greater than previously seen in transient expression assays using HIV-1 long terminal repeat chloramphenicol acetyltransferase gene constructs. In contrast to the defects in viral growth found in T-lymphocyte cell lines, several of the viruses containing TAR mutations were much less defective for gene expression and replication in activated peripheral blood mononuclear cells. These results indicate that maintenance of the TAR element is critical for viral gene expression and replication in all cell lines tested, though the cell type which is infected is also a major determinant of the replication properties of TAR mutant viruses.

The regulation of human immunodeficiency virus type 1 (HIV-1) gene expression is dependent on multiple *cis*-acting control elements in the long terminal repeat (LTR) (19) in addition to the transactivator protein, Tat (9, 15). A number of these regulatory elements including NF-κB (40), SP1 (23, 26), TATA (5, 36, 43), and TAR (49) have been demonstrated to modulate the level of *tat* activation. It is possible that cellular factors binding to the HIV-1 LTR assemble unique transcription complexes which are the targets for transcriptional activation by *tat*. Thus, a study of both the different regulatory elements in the HIV-1 LTR and the cellular proteins that bind to these elements is critical for a better understanding of cellular targets for *tat* activation.

One HIV-1 regulatory element, TAR, is critical for *tat* activation (49). TAR forms a stable stem-loop RNA structure (39, 42) that contains three critical elements. These include a 3-nucleotide bulge between +23 and +25 (4, 6, 10, 51, 52), a 6-nucleotide loop between +30 and +35 (4, 14, 16, 51, 52, 61), and the upper stem structure between +18 and +43 (14, 16, 24, 25, 52, 54). Tat binds directly to the TAR RNA bulge (6, 10, 11, 51, 58) while a cellular factor designated TRP-185/TRP-1 binds to the loop sequences (56, 61) and other proteins have been found to bind to the stem (18). In addition, TAR DNA serves as the binding site for a variety of proteins (17, 27, 29, 62) which may be involved in the generation of short transcripts from the HIV-1 LTR (48, 55). Heterologous constructs containing TAR fused to a variety of different promoters are capable of being activated by *tat*, indicating the critical role of this element (5, 39, 45, 48, 60). Thus, TAR is a complex regulatory element which is important in modulating *tat*-mediated gene expression from the HIV-1 LTR.

The function of the TAR element has been studied by transient expression assays of wild-type and mutant HIV-1 LTR templates in both the presence and the absence of *tat* (4, 14, 16, 24, 25, 49, 52, 54). In addition, transient transfection assays of wild-type and TAR mutant proviral constructs have also been used to demonstrate a critical role for TAR in regulating HIV-1 gene expression (22). However, it has not previously been possible to generate high-titer stocks of TAR mutant viruses to study the effects of these mutations on gene expression and growth properties. Viruses with mutations in other HIV-1 regulatory elements such as NF-κB and SP1 have been constructed, and the effects of these mutations on viral growth properties have been analyzed (35, 37, 44, 50). These studies indicated that viral growth properties were affected by both the specific mutation introduced and the cell type with which viral growth was analyzed.

The low levels of gene expression from viruses containing mutations in TAR prevent the generation of measurable levels of virus by previously described techniques. Though gene expression from HIV-1 TAR mutant proviral constructs can be induced by treatment of T-lymphocyte cell lines with phytohemagglutinin (PHA) and phorbol esters (22), this protocol to produce TAR mutant viruses is limited by the cytotoxicity of phorbol esters. Previous studies indicate that the adenovirus

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E1A and E1B proteins can induce gene expression from the HIV-1 LTR (32, 41). This activation requires the SP1 and TATA elements but is not markedly dependent on the TAR element (32). In this study, we utilized 293 cells, an E1A- and E1B-transformed human embryonic kidney cell line (21), to produce a variety of HIV-1 TAR mutants. These viruses contain mutations that either alter the TAR stem structure, delete the bulge, transpose the bulge to the opposite side of TAR, or change the sequence of the loop. We compared the growth kinetics of each of these viruses on several T-lymphocyte cell lines including H9 (46), Jurkat (59), and a Jurkat cell line constitutively expressing *tat* (7). In addition, we also tested the growth of these viruses on peripheral blood mononuclear cells (PBMCs). These studies demonstrate the importance of the HIV-1 TAR element in modulating viral gene expression and growth properties, though cell type differences are also critical in regulating the function of TAR.

MATERIALS AND METHODS

Plasmid construction. The proviral construct pBRDH1 contains a permutation of HIV-1 sequences at a unique *Mro*I located in the U3 region of the HIV-1 LTR. It was derived from the molecular clones SF2 *Mro*I(−156)-*Sph*I(+988), pBH10 *Sph*I(+988)-*Xho*I(+8486), and SF2 *Xho*I(+8486)-*Mro*I(+8982) (53). The complete *Mro*I DNA fragment was cloned into pBR322 at the unique *Mro*I site, cut with *Cla*I (end filled) and *Nru*I to remove pBR322 sequences, and closed with T4 DNA ligase. The complete molecular clone was not infectious when transfected into permissive cells unless it was first linearized with the restriction enzyme *Mro*I. After transfection into a permissive cell line, the linear fragments concatenate and express HIV-1. The LTR mutations were previously described (16, 61). A vector was constructed from an *Ava*I(−160) (end-filled)-*Sph*I(+988) DNA fragment from the HIV-1 SF2 isolate which was then cloned into pUC18 linearized with *Sma*I and *Sph*I. To construct each HIV-1 TAR mutant provirus, a *Pvu*I-*Sph*I DNA fragment containing each of the TAR mutants was ligated into pBRDH1 cut with the same restriction enzymes.

To insert the *neo* gene into pBRDH1, a *Sma*I site was introduced by M13 site-directed mutagenesis into pBRDH1. This destroyed the *nef* (pBH10) initiating methionine, and this construct was designated pBRDH2. Next, a *Bcl*I-*Nae*I (partial) DNA fragment from TN5 which contains the entire *neo* gene (2) was ligated into the pBSK vector (Stratagene) linearized with *Hin*dII. An *Eco*RV-*Xho*I DNA fragment from this construct was ligated into wild-type and mutant pBRDH2 constructs linearized with *Sma*I(+8385) and *Xho*I(+8486) to generate the pBRDH2-neo construct.

Cell lines, viruses, and infections. To generate stable cell lines producing wild-type and mutant HIV-1, the human embryonic kidney cell line 293 (21) was transfected by calcium phosphate precipitation with 20 μg of either *Mro*I-linearized wild type or the TAR mutant pBRDH2-neo plasmid. Three days posttransfection, the 293 cells were split 1:40 and maintained in Iscove's medium containing 5.0% newborn calf serum, 2.5% fetal bovine serum, 1% penicillin-streptomycin, and 1 mg of G418 (Geneticin; Bethesda Research Laboratories) per ml. The medium was changed every 4 days until foci appeared and grew to 2 mm in diameter. Cells were removed by using cloning wells, expanded, and assayed for HIV (p24) antigen (Ag). Cell-free supernatants were assayed for reverse transcriptase (RT) activity as previously described (47).

To produce supernatant for viral infection, freshly confluent 293 cells producing different HIV-1 mutants were grown for 12 to 16 h in RPMI containing 10% fetal bovine serum (heat inactivated), 1% glutamine, and 1% penicillin-streptomycin. The culture supernatant was removed, filtered through a 0.4-μm-pore-size membrane, assayed for RT activity, and used immediately to infect cells of either Jurkat (a human T-cell lymphocytic cell line) (59), Jurkat-*tat* (7) (the same cell line constitutively expressing the HIV transactivator protein, Tat), or H9 (a human cutaneous T-cell lymphoma) (46) or activated PBMCs. These cell lines were maintained in RPMI medium containing 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. For infection of PBMCs from an HIV-1-seronegative donor, cells were activated for 3 days with PHA (1 μg/ml) and maintained in the same culture medium supplemented with 30 U of interleukin-2 per ml (50).

For viral infection, 2×10^6 cells were incubated with filtered 293 supernatants containing 10^6 cpm of total ^{32}P-RT activity. Cells were incubated with viral supernatants for 8 to 12 h in 5 ml of culture medium. Next, the cells were pelleted at $400 \times g$, washed three times with 10 ml of culture medium, and resuspended in 10 ml of RPMI containing 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin. Cultures were split 1 to 7 every 3 or 4 days, and following centrifugation of aliquots of medium at $2{,}000 \times g$ to remove cells, they were assayed for RT activity and p24 Ag. To obtain G418-resistant Jurkat cell lines containing TAR mutant viruses, aliquots of infected and uninfected Jurkat cells were placed in culture medium containing 2 mg of G418 per ml at 4 weeks postinfection.

For single-cycle HIV-1 infections, approximately 1.5×10^6 Jurkat cells were incubated with 293 supernatants containing the equivalent of 2×10^6 cpm of total ^{32}P-RT activity. Cells were incubated for 2 h, and the samples were shaken every 15 min. The cells were spun at $400 \times g$, washed three times with 10 ml of culture medium, and resuspended in 0.5 ml of cell lysis buffer (760 mM guanidine hydrochloride, 10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl; pH 8.5). Each sample was frozen and thawed, incubated at 50°C in the presence of 25 μg of proteinase K for 1 h, and extracted once with phenol-chloroform, and the nucleic acids were precipitated with 2 volumes of ethanol.

RT assay and ELISA for p24 Ag. A mini-RT assay was used to analyze the HIV-1-infected culture supernatants (47). Briefly, 10 μl of cell free supernatant was mixed for 90 min at 37°C with a reaction cocktail containing 50 mM Tris (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 0.05% Nonidet P-40, 250 ng of poly(rA) oligo(dT)$_{12-18}$ (Pharmacia), and 0.5 μCi of [^{32}P]dTTP (PB10167; Amersham). The reaction mixtures were spotted onto DEAE paper (NA45; Schleicher & Schuell) and washed three times at room temperature in 2× SSC (0.3 M NaCl, 0.3 M sodium citrate; pH 7.0). Incorporated counts were measured by liquid scintillation. Cell-free supernatants were assayed for p24 Ag at a detection limit of 10 pg/ml by an enzyme-linked immunosorbent assay (ELISA), HIVAG-1 (Abbott), according to the manufacturer's instructions.

PCR analysis. Chromosomal DNAs from 293 cell lines and G418-selected Jurkat cells were obtained with Qiagen DNA extraction reagents and used in subsequent PCR analysis. The chromosomal DNA from either HIV-1-producing 293 cell lines or G418-selected Jurkat cells was used in PCR mixtures containing 0.5 μg of DNA and 0.5 μg of each oligonucleotide [5′-CCCAAACAAGACAAGAGATTGA-3′ (−436/−415, sense) and 5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′ (+242/+219, antisense)]. Each sample was subjected to 35 cycles at 55°C for annealing, 72°C for synthesis, and 95°C for denaturing with 1 min at each temperature. The resulting DNA fragments, which included most of the 5′ LTR and the

primer binding site, were ligated into the vector pCRII (Invitrogen) and sequenced with Sequenase reagents (U.S. Biochemicals).

PCR conditions used to analyze the single-cycle infections and G418-selected Jurkat cells containing TAR mutant viruses were previously described (64). Briefly, two oligonucleotide primers, 5′-GCTAACTAGGGAACCCACTGC 3′ [+44/+64, sense)] and 5′-CTGCTAGAGATTTTTCCACACTGAC-3′ [(+183/+159, antisense)] were used to amplify a 139-bp fragment from the R-U5 junction of the HIV-1 LTR. Approximately 0.1 μg of total DNA isolated from the infected cells was subjected to 25 cycles of PCR using 30 ng of ^{32}P-end-labeled sense primer (5×10^8 cpm/μg) along with 100 ng of unlabeled antisense primer and reactions were incubated first at 65°C for 2 min and then at 92°C for 1 min. The HIV-1 standards used represent a molecular SF-2 (53) proviral clone present at either 0, 10, 10^2, 10^3, or 10^4 copies containing 100 ng of sonicated herring sperm carrier DNA. A pair of oligonucleotide primers complementary to the first exon of the human β-globin gene nucleotide which generates a 110-bp band between positions 14 to 33 (5′-ACACAACTGTGTTCAC TAGC-3′) and 123 to 104 (5′-CAACTTCATCCACGTTCA CC-3′) was used as a control for the total amount of DNA in each reaction mixture. PCR products were resolved on a 6% polyacrylamide gel and subjected to autoradiography.

Northern (RNA) analysis. Total RNA was extracted from Jurkat and 293 cells with RNAsol B according to the manufacturer's instructions (Biotecx Laboratories). An agarose gel containing 1% formaldehyde was used in electrophoresis of 30 μg of total RNA at 100 V for 3 h. The separated RNA was transferred overnight to nitrocellulose by the capillary method, and the filter was baked for 2 h at 80°C. A *Bam*HI(+8050)-*Sma*I(+8385) DNA fragment from pBRDH2 was labeled by random priming (Boehringer Mannheim) with [^{32}P]dCTP, which is capable of hybridizing to all spliced and unspliced HIV-1 RNA transcripts. Blots were prehybridized and then hybridized overnight at 47°C in 1× hybridization solution (Bethesda Research Laboratories) containing 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and 10^6 cpm of denatured probe per ml at 47°C. The filter was then washed with 2× SSC–0.1% SDS at room temperature and then with 0.2× SSC containing 0.1% SDS at 65°C for 15 min prior to autoradiography.

Western immunoblot analysis. Whole-cell supernatants were prepared from 293 cells and G418-selected Jurkat cell lines, each containing different HIV-1 TAR mutants. A pellet containing 10^7 cells was frozen and thawed twice and then treated with 1,000 U of micrococcal nuclease (Worthington Biochemical) at 37°C for 30 min. Each cell pellet was resuspended in 1× Laemmli buffer at a concentration of 10^8 cells per ml and heated to 95°C for 10 min. Whole-cell extract prepared from 5×10^5 Jurkat or 1×10^6 293 cells was subjected to electrophoresis on a 12% polyacrylamide gel, transferred to nitrocellulose, and probed with a 1:5,000 dilution of purified human anti-HIV-1 immunoglobulin G (IgG) (NIH AIDS Research and Reagent Program no. 192). A second antibody, horseradish peroxidase-conjugated rabbit anti-human IgG (Amersham) which was diluted 1:2,000, was then used for enhanced chemiluminescence detection (Amersham).

RESULTS

Construction of TAR mutant viruses. The preservation of the upper stem structure of TAR RNA between +18 and +43 is critical for *tat* activation (4, 14, 16, 24, 25, 52, 54). In addition, the upper portion of TAR RNA contains two important regulatory elements that are also important for *tat* activation. One is the 3-nucleotide bulge between positions +23 and +25 (4, 6, 10, 51, 52), and the other is a 6-nucleotide loop between positions +30 and +35 (4, 14, 16, 51, 52, 61). Previously, we constructed a variety of TAR mutations that disrupted the upper stem, [(+19/+22) and (+40/+43)], changed the primary sequence of the loop [(+31/+34)], altered the bulge [(+25) and Δ(+23/+25)], transposed the bulge to the opposite side of the stem structure [Δ(+23/+25)/(+37/+39)], disrupted both the upper and lower stem structures [(+11/+14)/(+40/+43)], or restored stem structure but altered the primary sequence [(+19/+22)/(+40/+43)] (Fig. 1). HIV LTR chloramphenicol acetyltransferase (CAT) gene reporter plasmids containing these or similar mutations resulted in a 3- to 20-fold decrease in *tat*-mediated gene expression compared with that of a wild-type construct (4, 14, 16, 24, 25, 49, 52, 54). However, another construct, (+19/+22)/(+40/+43), in which stem secondary structure was maintained but the primary sequence of TAR was altered, was not defective for *tat*-induced gene expression (16, 61).

Each of these TAR mutations was inserted into the proviral vector pBRDH1, which contains the entire HIV-1 genome permuted at a unique *Mro*I site in the HIV-1 LTR. This construct contains only one copy of HIV-1 LTR, but it is not infectious unless linearized with the restriction enzyme *Mro*I and transfected into permissive cells. The DNA is efficiently ligated when transfected into mammalian cells, generating concateramized fragments that express viral RNAs (63). Though significant quantities of wild-type virus were generated by this procedure, attempts to produce similar levels of TAR mutant viruses in a variety of human cell lines, including HeLa, Jurkat, H9, and Jurkat-*tat*, were unsuccessful (data not shown). This was likely due to the marked defects in gene expression seen with these TAR mutants.

We attempted to overcome the low level of gene expression seen with HIV-1 TAR mutants by transfecting these constructs onto cell lines which expressed the adenovirus transactivator E1A. E1A can markedly increase HIV-1 gene expression in the absence of *tat* in a manner which is not strictly dependent on TAR structure (32). For these experiments, we used the human embryonic kidney cell line 293, which stably expresses E1A in addition to a second adenovirus protein, E1B. Transfection of 20 μg of linearized HIV-1 proviral DNA containing each of the TAR mutations into 293 cells produced low levels of secreted p24 Ag (data not shown). However, no detectable RT activity was identified, and passage of these transfected 293 cells resulted in the complete loss of viral gene expression, indicating that gene expression from these mutants was transient.

Next, we sought to determine if we could isolate stable 293 cell lines which express HIV-1 TAR mutants. To perform these experiments, we inserted the neomycin phosphotransferase II, or *neo*, gene (2) into the *nef* open reading frame so that cells containing these viruses could be selected with G418. This was possible because the *nef* gene is not essential for HIV-1 replication in tissue culture (15, 57). Oligonucleotide-directed mutagenesis was performed to eliminate the *nef* initiating methionine prior to the insertion of the *neo* gene into the *nef* open reading frame of wild-type HIV-1 and each of the TAR mutants. Following the transfection of HIV-1 proviral constructs containing TAR mutations into 293 cells, we were able to select cell lines with G418 that contained each of these viruses. The procedure used to generate stable 293 cell lines containing each of the HIV-1 TAR mutants is diagrammed in Fig. 2.

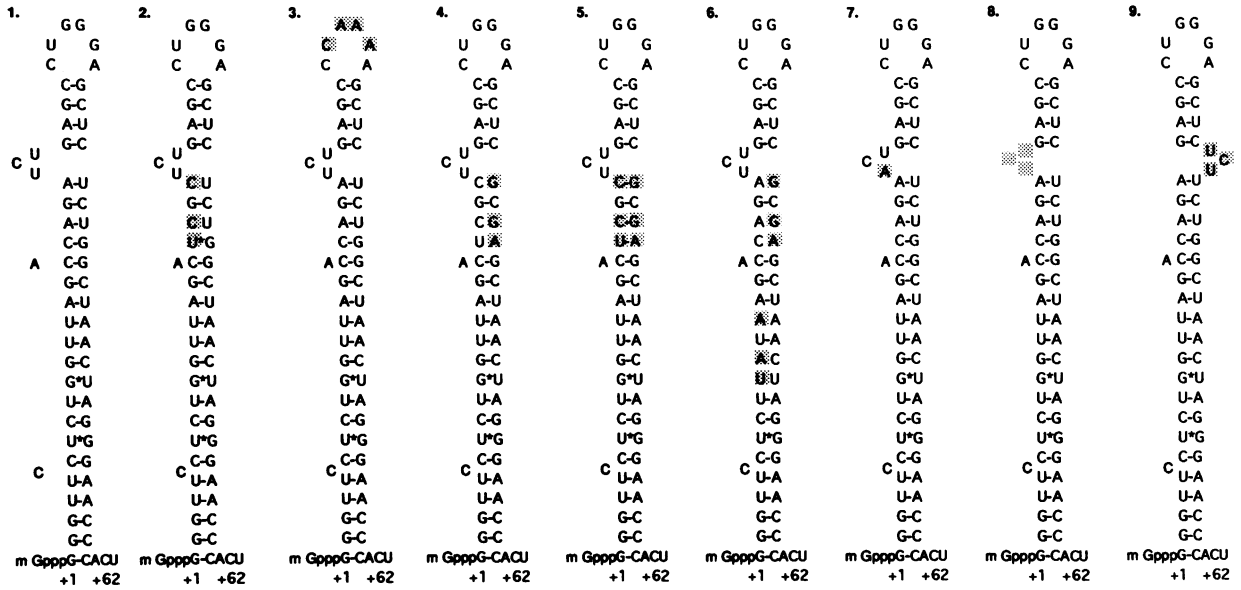

FIG. 1. Schematic of different HIV-1 LTR TAR mutations. A portion of the HIV-1 LTR TAR element extending from +1 to +62 is shown. Positions of mutations in TAR RNA are indicated for the following: 1, wild type; 2, (+19/+22); 3, (+31/+34); 4, (+40/+43); 5, (+19/+22)/(+40/+43); 6, (+11/+14)/(+40/+43); 7, (+23); 8, Δ(+23/+25); and 9, Δ(+23/+25)/(+37/+39).

293 cells can produce infectious HIV-1 TAR mutants. Supernatants from each of the 293 cell lines containing either wild-type virus or TAR mutants were assayed for p24 Ag and RT activity. The 293 isolates expressing TAR mutant provirus (+19/+22), (+31/+34), or (+23) produced 25 to 35 ng of released p24 Ag per ml; the cell lines expressing (+40/+43), (+11/+14)/(+40/+43), or Δ(+23/+25) produced 50 to 60 ng/ml; and cells expressing Δ(+23/+25)/(+37/+39), (+19/+22)/(+40/+43), or the wild-type virus produced 95 to 130 ng of p24 Ag per ml (Fig. 3A). The ^{32}P-RT activity in the same culture supernatants ranged from 1.4×10^6 to 5.5×10^6 cpm/ml, as indicated in Fig. 3B. The amounts of secreted p24 Ag correlated well with the RT activity such that a ratio of ^{32}P-RT activity to p24 Ag was approximately 40 to 53 [(cpm/μl)/(p24 ng/ml)]. Northern and Western blot analyses were also performed and confirmed the presence of virus-specific RNAs and proteins in each of the 293 cell lines (data not shown). It was also critical to determine whether any DNA rearrangements occurred in the LTRs of these proviruses. To confirm the integrity of the integrated provirus in each 293 cell line, PCR amplification was performed with specific oligonucleotide primer pairs corresponding to both the 5′ and the 3′ LTRs. The primers amplified either a region extending from −436 to +242 which included the 5′ LTR and the primer binding site or a region from +9146 to +9722 which included both the polypurine tract and the 3′ LTR. The primer pairs produced the expected 678- and 576-bp DNA fragments, respectively, and the integrity of each mutation was confirmed by DNA sequence analysis of the PCR-amplified fragments (data not shown). Thus, we were able to obtain 293 cell lines containing each of the TAR mutant proviruses whose sequences are shown in Fig. 1.

HIV-1 TAR mutants do not exhibit defects in viral entry or initiation of reverse transcription. The T-lymphocyte cell lines H9 and Jurkat were infected with 10^6 ^{32}P-RT cpm equivalents of either wild-type HIV-1 produced by 293 cells or HIV-1 isolates IIIb (46) and SF-2 (53). There were no significant differences in the replication kinetics among these three viruses, indicating that the *neo* gene did not alter viral replication properties (data not shown). Next, it was important to determine whether alterations in TAR structure affected the ability of viruses to infect T lymphocytes or to undergo reverse transcription. To directly test whether TAR mutations altered either viral cell entry or the initiation of reverse transcription, single-cycle infections of Jurkat cells with wild-type and TAR mutant viruses were performed and analyzed by PCR. Jurkat cells were incubated with 2×10^6 ^{32}P-RT cpm equivalents of wild-type virus or the TAR mutants (+19/+22), (+31/+34), (+40/+43), (+19/+22)/(+40/+43), (+11/+14)/(+40/+43), and Δ(+23/+25). As controls, Jurkat cells were incubated with either heat-inactivated wild-type virus or supernatant from uninfected cells. After 2 h of infection at 37°C, the cells were washed three times and approximately 0.1 μg of total DNA from these cells was subjected to PCR analysis.

^{32}P-labeled oligonucleotide primer pairs (+44/+64, sense) and (+183/+159, antisense), complementary to the R and U5 regions of the HIV-1 LTR, respectively, should amplify a specific band of 139 bp. A species of the expected size was amplified from Jurkat cells infected with wild-type virus (Fig. 4A, lane 7). As shown in Fig. 4A, PCR amplification of DNA from Jurkat cells infected with the TAR mutants (+19/+22) (lane 1), (+31/+34) (lane 2), (+40/+43) (lane 3), (+19/+22)/(+40/+43) (lane 4), (+11/+14)/(+40/+43) (lane 5), and (+23/+25) (lane 6) gave the same-size species of comparable intensity. Heat inactivation of the wild-type virus reduced the signal approximately 50-fold (Fig. 4A, lane 8), and no species was detected in mock-infected cells (Fig. 4A, lane 9). Since PCR is specific for DNA targets, all of the viruses likely have relatively equal abilities to gain entry to the cell and initiate reverse transcription. Further experiments with these viruses, both in the presence and in the absence of RT inhibitors, are under way to characterize potentially subtle defects in reverse transcription. Titration of a molecular HIV-1 provirus present at either 0, 10, 10^2, 10^3, or 10^4 copies (Fig. 4A, lanes 10 to 14) demonstrated that the PCR was linear and quantitative. Oligonucleotide primers specific to the first intron of the human

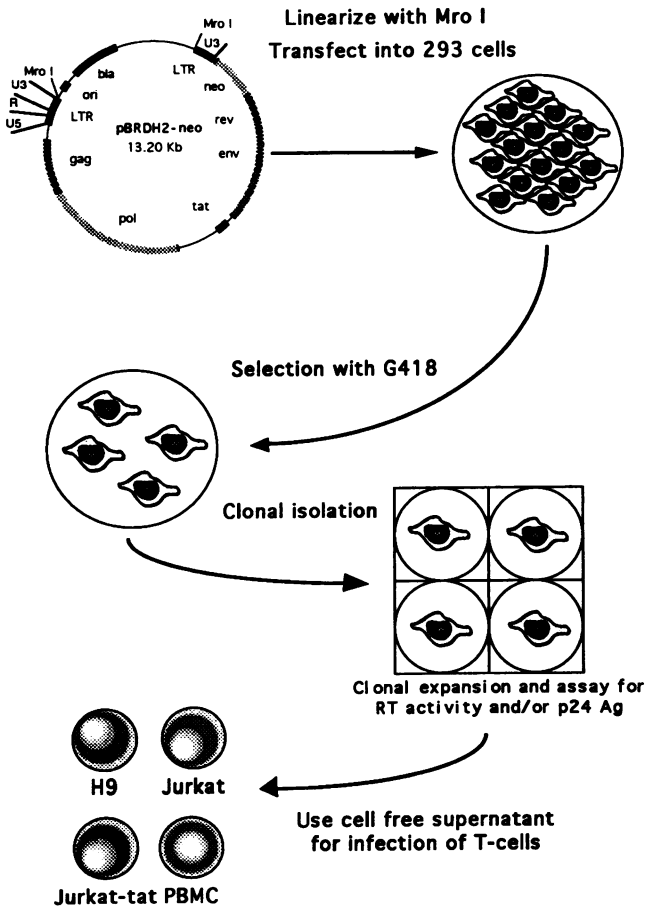

FIG. 2. Schematic of procedure for production of HIV-1 TAR mutants. An HIV-1 molecular proviral clone (pBRDH2-neo) was linearized with the restriction enzyme *Mro*I and transfected into 293 cells, and the cells were split into medium containing G418. Foci were isolated and expanded, and supernatants were assayed for the production of p24 Ag and RT activity prior to their use for infection of both T-cell lines and PBMCs.

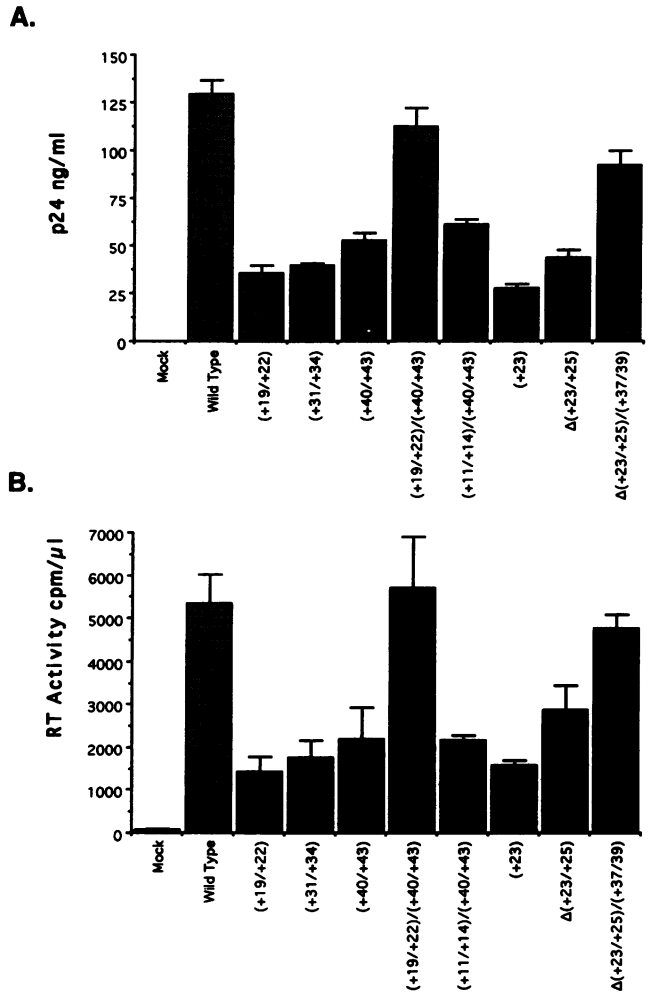

FIG. 3. Assays of viral gene expression in 293 cells. The amounts of secreted p24 Ag (A) and RT (B) detected in culture supernatants following an overnight incubation in fresh medium are indicated for mock-infected 293 cells and 293 cells containing wild-type HIV-1 or the indicated TAR mutants. Results were obtained for 3 consecutive days from the same freshly confluent plate of 293 cells, and the standard deviations were calculated.

β-globin gene, which amplify a specific 110-bp band, were included as a control for the amount of DNA in each PCR analysis. There were no significant differences in the intensity of this band among the different samples (Fig. 4B, lanes 1 to 9). PCR amplification as shown in Fig. 4B for 0 (lane 10), 2×10^{-3} (lane 11), 2×10^{-2} (lane 12), and 0.2 (lane 13) μg of Jurkat chromosomal DNA normalized with herring sperm DNA demonstrated that the amplification of the β-globin gene was linear and quantitative. These results indicate that TAR mutant viruses do not exhibit defects in their ability to either infect lymphocytes or initiate reverse transcription.

TAR mutant viruses are defective for replication in T-lymphocyte cell lines. To determine if mutation of the TAR element influenced viral gene expression and growth kinetics, supernatants containing viruses produced from the cloned 293 cell lines were used to infect the T-lymphocyte cell lines H9 and Jurkat. Subsequent virus production was monitored by assays for the expression of both p24 Ag and RT activity. For each infection, 2×10^6 cells were infected with 10^6 ^{32}P-RT cpm of either wild-type or TAR mutant viruses. The p24 Ag data showed that both the wild-type and the TAR stem restoration mutant virus, (+19/+22)/(+40/+43), replicated efficiently in H9 and Jurkat cell lines (Fig. 5A and B). The p24 expression increased rapidly over the first week and reached maximal expression in approximately 12 to 14 days. Cultures maintained for 48 to 50 days remained positive for p24 Ag (Fig. 5A and B). In sharp contrast, infection of either H9 or Jurkat cells with TAR mutant viruses (+19/+22), (+31/+34) (+40/+43), (+11/+14)/(+40/+43), (+23), Δ(+23/+25), and Δ(+23/+25)/(+37/+39) produced a low (20- to 100-pg/ml), transient release of p24 Ag after 4 days (Fig. 5A and B). However, the levels of p24 by day 7 were two- to fourfold lower, ranging from 15 to 20 pg/ml, and decreased markedly by 10 days of infection. Jurkat cells infected with the TAR mutant (+23), which contains a point mutation in the bulge, produced the largest burst of p24 Ag, which was maintained for 14 days but decreased approximately two- to threefold after each passage. These data indicated that most of the HIV-1 TAR mutants were capable of only transient expression of low levels of p24 Ag over a 50-day period of infection.

To test if the low levels of gene expression from the different

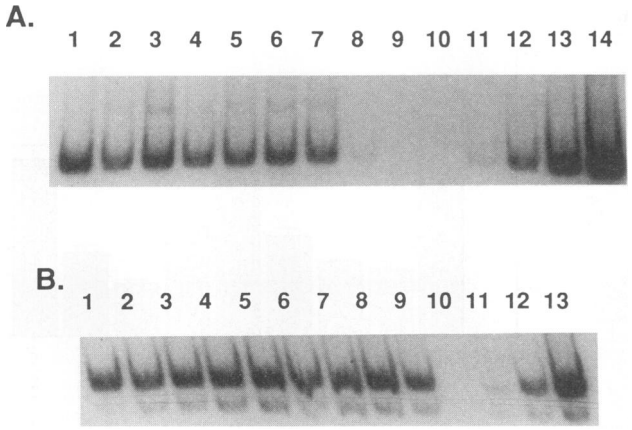

FIG. 4. PCR analysis of single-cycle infection of Jurkat cells by wild-type and TAR mutant viruses. A 2-h infection of 10^6 Jurkat cells was performed with 2×10^6 cpm of ^{32}P-RT obtained from 293 cell lines containing either the wild type or TAR mutants. Total DNA was isolated from the infected cells and subjected to 25 cycles of PCR with ^{32}P-labeled specific primers. (A) The presence of a 139-bp specific amplified band from the HIV-1 LTR was determined for the following: lane 1, (+19/+22); lane 2, (+31/+34); lane 3, (+40/+43); lane 4, (+19/+22)/(+40/+43); lane 5, (+11/+14)/(+40/+43); lane 6, Δ(+23/+25); lane 7, wild-type virus; lane 8, heat-inactivated wild-type virus; and lane 9, uninfected Jurkat cells. HIV-1 standards which represent a molecular clone present at 0 (lane 10), 10 (lane 11), 10^2 (lane 12), 10^3 (lane 13), or 10^4 (lane 14) copies are also shown. (B) The 110-bp specific band produced by PCR with a primer pair corresponding to the human β-globin gene was used as a control for the amount of DNA in panel A (lanes 1 to 9). Also shown are results of PCR analysis using the β-globin primer pair with either 0 (lane 10), 0.02 (lane 11), 0.1 (lane 12), or 0.5 (lane 13) μg of Jurkat chromosomal DNA.

TAR mutant viruses were due to the lack of expression of the Tat protein, a Jurkat cell line constitutively expressing Tat (7) was infected with the same panel of viruses (Fig. 5C). As expected, the wild type and the TAR stem restoration mutant, (+19/+22)/(+40/+43), replicated in these cells with kinetics similar to those for parental Jurkat cells. The maximal p24 Ag expression from these viruses in this cell line was roughly twofold higher than that in the Jurkat cells lacking *tat* (1,300 ng/ml [Fig. 5C] versus 600 ng/ml [Fig. 5B]). However, the transient levels of p24 Ag for several TAR mutant viruses on day 4 were four- to fivefold lower than observed when these viruses were used to infect H9 or Jurkat cells (Fig. 5B and C). One mutant, Δ(+23/+25), produced no detectable transient burst, while another TAR mutant, (+40/+43), expressed marginally detectable p24 Ag (10 to 15 pg/ml) for 30 days (Fig. 5C). A virus harboring a point mutation in the bulge, (+23), expressed 600 pg of p24 Ag per ml by day 4 (50% of wild-type expression), but this expression decreased by 10-fold over 52 days (Fig. 5C). Similar results were seen when reverse transcription assays were used (data not shown). These data demonstrated that the constitutive expression of *tat* could not compensate for the deleterious effect of these altered TAR structures on HIV-1 gene expression.

Viral growth kinetics in PBMCs. Previous data indicated that activation of T-lymphocyte proliferation markedly increased the gene expression of HIV-1 TAR mutants (22). This effect was likely due to the stimulation of binding of NF-κB proteins to the HIV-1 enhancer (22, 40). Thus, we assayed the growth properties of TAR mutant viruses on PBMCs that were stimulated with PHA and interleukin-2. PBMCs were obtained

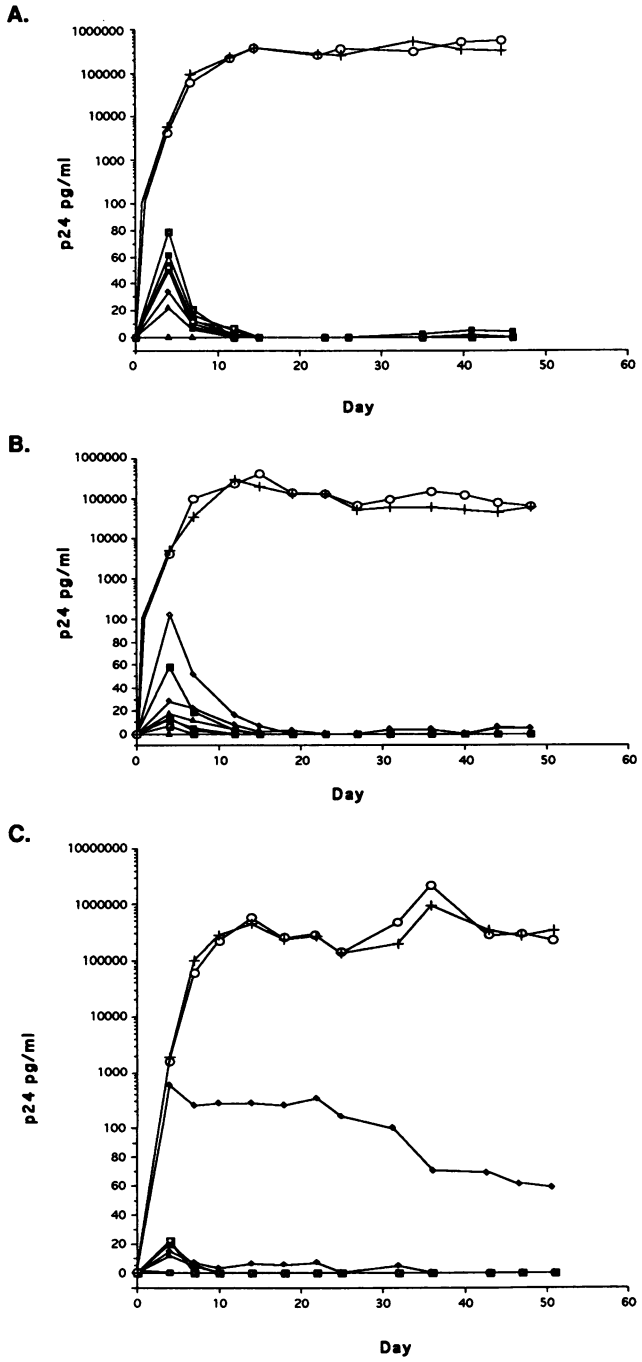

FIG. 5. Assay of p24 antigen following infection of human T lymphocytes with wild-type virus and TAR mutant viruses. H9 (A), Jurkat (B), or Jurkat-*tat* (C) cells were infected with 293 mock supernatant (△) or supernatants containing approximately 10^6 ^{32}P-RT cpm of either wild type (+), (+19/+22) (⊡), (+31/+34) (▲), (+40/+43) (◆), (+19/+22)/(+40/+43) (○), (+11/+14)/(+40/+43) (■) (+23) (◇), Δ(+23/+25) (■), or Δ(+23/+25)/(+37/+39) (□). Infections were performed three times and viral expression was determined by an ELISA for secreted p24 Ag present in cell-free culture supernatant. Results were similar in each of the three experiments.

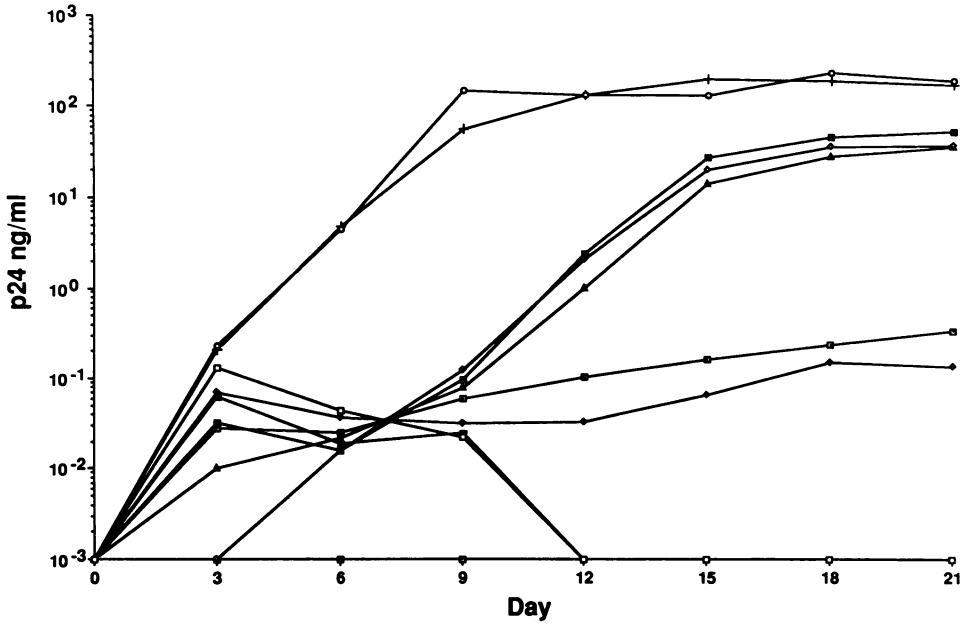

FIG. 6. Assay of p24 antigen following infection of human PBMCs with wild-type and TAR mutant viruses. A total of 2×10^6 PHA-activated PBMCs were infected with 10^6 cpm of ^{32}P-RT for 2 h at 37°C, and the cells were washed three times and maintained in complete medium supplemented with 30 U of interleukin-2 per ml. The p24 Ag levels were determined by ELISA every third day for mock infection (△), infection with wild-type HIV-1 (+), or infection with TAR mutant (+19/+22) (⊡), (+31/+34) (▲), (+40/+43) (◆), (+19/+22)/(+40/+43) (○), (+11/+14)/+40/+43) (■), (+23) (◇), (+23/+25)/(+37/+39) (□) or Δ(+23/+25) (■). Infections were performed three times with similar results.

from an HIV-1-seronegative donor and activated for 3 days in culture medium supplemented with 1 μg of PHA per ml. The cells were washed three times, and 10^6 cells were infected with 10^6 ^{32}P-RT U of either wild-type or TAR mutant viruses. The cells were infected for 2 h and then washed three times with culture medium and resuspended in complete medium supplemented with 30 U of interleukin-2 per ml (50). In contrast to the results found with T-cell lines, TAR mutant viruses displayed differential growth kinetics in activated PBMCs. As noted for T-lymphocyte cell lines, the virus containing the TAR stem restoration mutant, (+19/+22)/(+40/+43), replicated as well as wild-type virus (Fig. 6). However, viruses containing mutations that altered either the loop sequence [(+31/+34)] or the bulge sequence [(+23)] or deleted the bulge altogether [Δ(+23/+25)] displayed somewhat reduced growth kinetics and five- to eightfold decreases in p24 Ag by day 18 (Fig. 6). This was in contrast to the results seen with these viruses when infections were performed on T-lymphocyte cell lines, for which it was found that these viruses were much more defective (Fig. 5). Viruses with mutations that altered TAR RNA secondary structure, (+19/+23) and (+40/+43), produced 800- to 1,400-fold less p24 Ag by day 18 than wild-type virus. Viruses containing mutations that disrupted the TAR RNA secondary structure [(+11/+14)/(+40/+43)] or transposed the bulge to the opposite side of the TAR RNA stem [Δ(+23/+25)/(+37/+39)] produced p24 Ag transiently, but it was not detectable (sensitivity threshold is 10 pg/ml) by day 18 (Fig. 6). RT assays were also performed, and though the levels for most of the TAR mutants were low compared with the wild type, they correlated well with the amount of p24 Ag (data not shown). These results indicate that several of the TAR mutants were not as defective for gene expression in stimulated PBMCs as they were in T-lymphocyte cell lines. However, several of the TAR mutants were unable to replicate in activated PBMCs, indicating that TAR was critical for viral growth in these cells.

TAR mutant viruses demonstrate defective transcription. Because viruses used in this study contained the *neo* gene, cells containing an integrated provirus could be resistant to the toxicity of G418. Even though we could not detect significant levels of p24 Ag in Jurkat cells infected with different TAR mutant viruses, we tested whether it was possible to obtain populations of Jurkat cells containing the different TAR mutant viruses. G418 was added to aliquots of both HIV-1-infected and uninfected Jurkat cells at 28 days postinfection. Jurkat cells infected with either the wild type or each of the TAR mutant viruses were drug resistant in times ranging from 2 weeks for the wild type and the TAR stem restoration mutant to 4 to 5 weeks for the other TAR mutants. No viable Jurkat cells were observed in the mock-infected cultures treated with G418. Chromosomal DNA purified from these HIV-1-infected cells were subjected to PCR with the oligonucleotide primer pairs (−436/−415, sense) and (+242/+219, antisense), which were specific for the 5′ LTR. The 678-bp DNA fragments generated by PCR for each of the HIV-1 isolates were subjected to DNA sequence analysis, and in each case the correct TAR mutation was confirmed (data not shown).

To assess the level of viral expression in these G418-selected Jurkat cell lines, the amounts of p24 Ag and RT detected in cell-free supernatants from three consecutive passages of each cell line were determined (Fig. 7). Jurkat cells infected with the wild type or TAR mutant virus (+19/+22)/(+40/+43) produced 200 to 400 ng of p24 Ag per ml, while Jurkat cells harboring other TAR mutant proviruses produced substantially less p24 Ag (Fig. 7A). The levels of p24 Ag in these different cells ranged from 100 to 500 pg/ml for (+19/+22), 400 to 900 pg/ml for (+31/+34), 1 to 2 ng/ml for (+40/+43), 70 to 600 pg/ml for (+11/+14)/(+40/+43), and 400 to 800 pg/ml

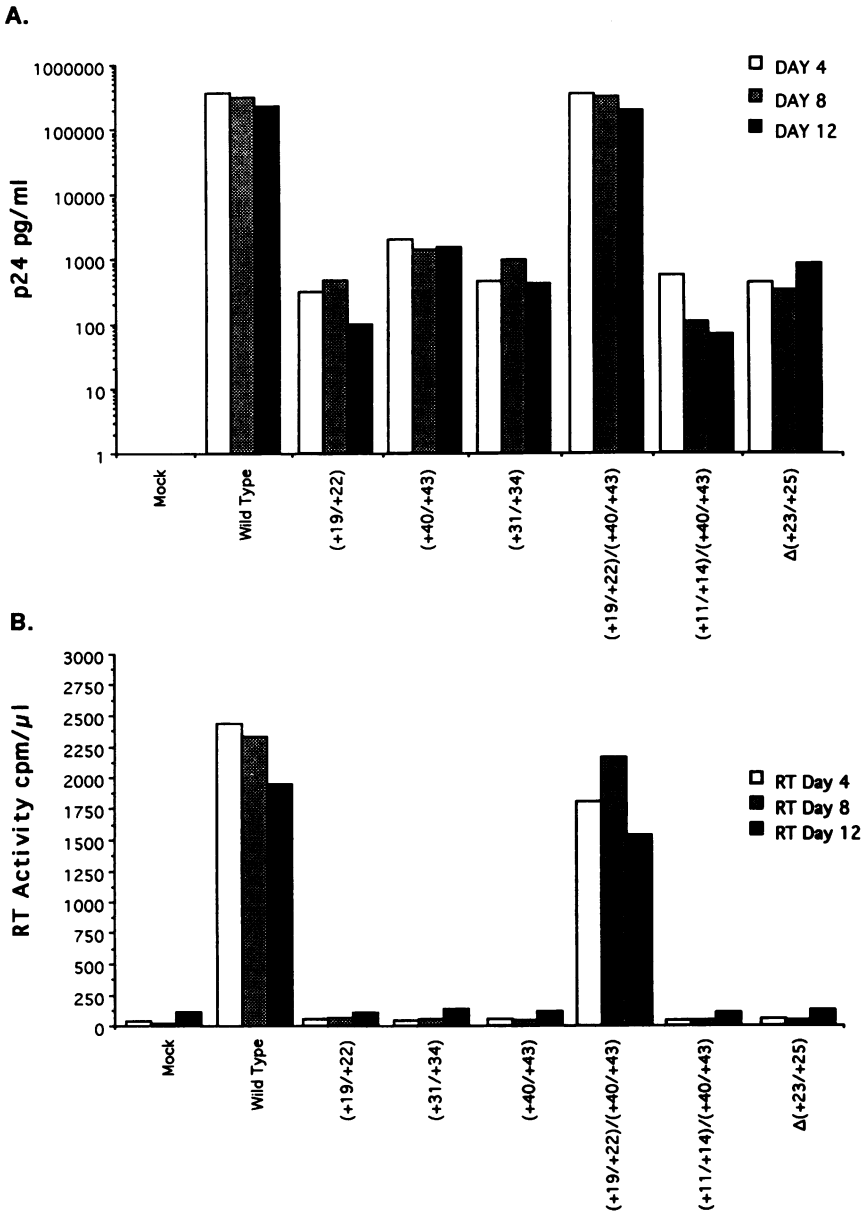

FIG. 7. Assay of p24 antigen levels following isolation of G418-resistant Jurkat cells containing TAR mutant viruses. Approximately 4×10^5 G418-resistant Jurkat cells containing different HIV-1 TAR mutants were grown for 4 days and then assayed before each passage for p24 Ag and RT levels. (A) Amounts of secreted p24 Ag in cell-free culture supernatants following passage 1 (4 days), passage 2 (8 days), and passage 3 (12 days) for cells either mock infected or infected with HIV-1 wild type or TAR mutants as indicated. (B) ^{32}P-RT activity detected in the same culture supernatants.

for Δ(+23/+25) (Fig. 7A). The same viral supernatants were also assayed for RT activity (Fig. 7B). As expected, only supernatants from the wild type or (+19/+22)/(+40/+43) had detectable RT activity (Fig. 7B). All other TAR mutant viruses, including (+19/+22), (+31/+34), (+40/+43), (+11/+14)/(+40/+43), and Δ(+23/+25), produced no detectable RT activity.

A Northern blot which contained 30 μg of total RNA isolated from each G418-selected Jurkat cell line was probed with an HIV-1 DNA fragment extending from nucleotides 8050 to 8385 to detect both spliced and unspliced HIV-1 RNA transcripts. The Northern blot in Fig. 8A showed a decrease in the steady-state levels of RNA with the TAR mutant proviruses (+19/+22) (lane 3), (+31/+34) (lane 4), (+40/+43) (lane 5), (+11/+14)/(+40/+43) (lane 7), and Δ(+23/+25) (lane 8) compared with those of either the wild-type virus (lane 2) or the TAR mutant virus (+19/+22)/(+40/+43) (lane 6). An overnight exposure was sufficient to identify the 2.8-, 5.1-, and 10-kb HIV-1-specific RNA transcripts (12, 13, 31) in Jurkat cells containing the wild-type or (+19/+22)/(+40/+43) proviruses (Fig. 8B), while a longer exposure was needed to detect these transcripts with the other TAR mutants (Fig. 8A). However, we note that the 5.1-kb transcript was only faintly visible (Fig. 8A). HIV-1 transcripts were not detected in

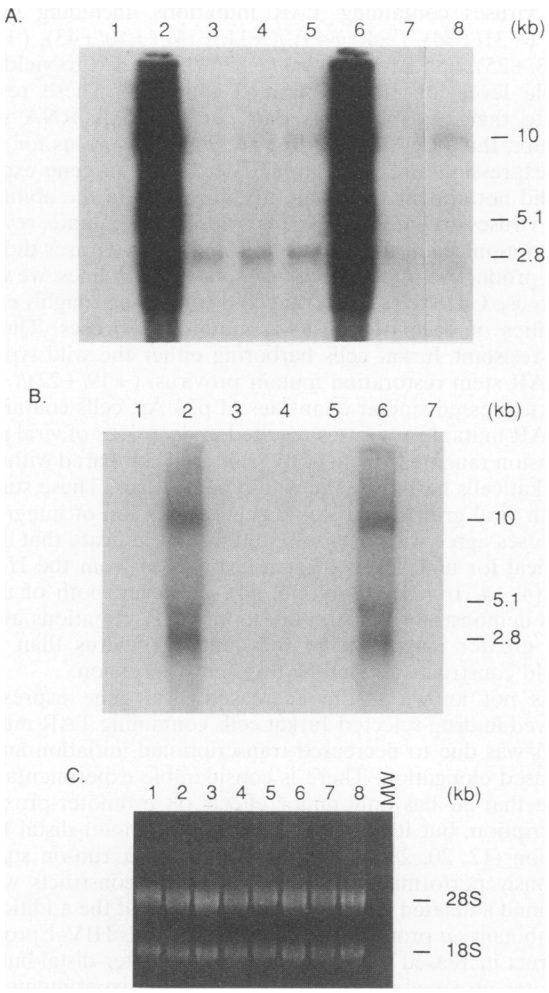

FIG. 8. Northern analysis of RNA isolated from G418-resistant Jurkat cells containing TAR mutant viruses. (A and B) Northern blot analysis of 30 μg of total RNA from uninfected cells (lanes 1) or cells infected with either wild-type HIV-1 (lanes 2) or TAR mutants (+19/+22) (lanes 3), (+31/+34) (lanes 4), (+40/+43) (lanes 5), (+19/+22)/(+40/+43) (lanes 6), (+11/+14)/(+40/+43) (lanes 7), and Δ(+23/+25) (lane 8) was performed with an HIV-1 probe extending from nucleotides 8050 to 8385. Six-day (A) and overnight (B) exposures are shown. (C) The 28S and 18S rRNA species from an ethidium bromide-stained 1% formaldehyde gel used for the Northern analysis are also shown. Lane MW, molecular size markers.

uninfected Jurkat cells (Fig. 8A and B, lanes 1), nor were there were significant differences in the amount of RNA loaded for these samples (Fig. 8C).

Whole-cell lysates were prepared from each of the G418-selected Jurkat cell lines for Western blot analysis using purified human anti-HIV-1 IgG (Fig. 9). No proteins were detected in lysates made from uninfected Jurkat cells (Fig. 9, lane 1). Three predominant proteins of 55, 41, and 24 kDa were detected in lysates prepared from all HIV-1-infected Jurkat cells. As shown in Fig. 9, the TAR mutant proviruses (+19/+22) (lane 3), (+31/+34) (lane 4), (+40/+43) (lane 5), (+11/+14)/(+40/+43) (lane 7), and Δ(+23/+25) (lane 8) made substantially less of these proteins than either the wild type (lane 2) or mutant (+19/+22)/(+40/+43) (lane 6). The

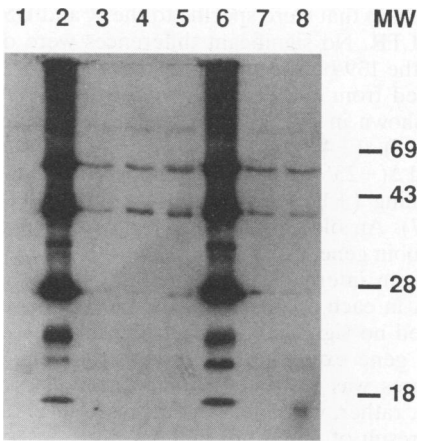

FIG. 9. Western blot analysis of cell lysates prepared from G418-resistant Jurkat cells containing TAR mutant viruses, performed with anti-HIV-1 IgG and detected by enhanced chemiluminescence. Whole-cell lysates were prepared from uninfected Jurkat cells (lane 1) or Jurkat cells infected with HIV-1 wild type (lane 2) or TAR mutants (+19/+22) (lane 3), (+31/+34) (lane 4), (+40/+43) (lane 5), (+19/+22)/(+40/+43) (lane 6), (+11/+14)/(+40/+43) (lane 7), and Δ(+23/+25) (lane 8). MW, molecular weight (indicated in kilodaltons).

levels of viral proteins were consistent with the levels of steady-state RNA detected for each proviral construct (Fig. 8).

One possible explanation for the decrease in the levels of viral RNA and protein in the G418-resistant Jurkat cells harboring TAR mutant viruses was that they contained a substantially lower proviral load than was found in cells containing wild-type virus. PCR analysis of chromosomal DNA isolated from each of the Jurkat cell lines was performed with ^{32}P-labeled primer pairs (+44 to +64, sense) and (+183 to

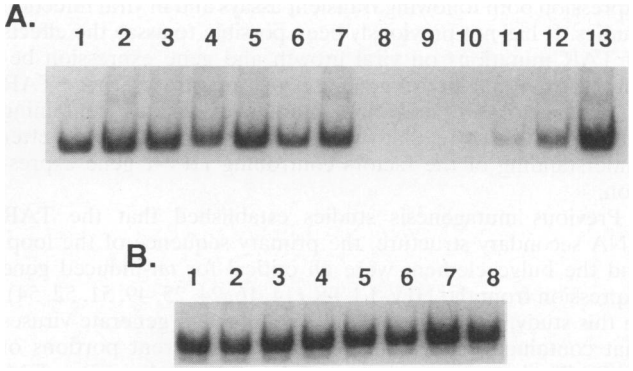

FIG. 10. PCR analysis of proviral load in chromosomal DNA isolated from G418-selected Jurkat cells. (A) Approximately 0.1 μg of chromosomal DNA isolated from G418-selected Jurkat cells was subjected to 25 cycles of PCR with ^{32}P-labeled specific primers for the HIV-1 R and U5 regions. The presence of the 139-bp specific amplified band is indicated for (+19/+22) (lane 1), (+31/+34) (lane 2), (+40/+43) (lane 3), (+19/+22)/(+40/+43) (lane 4), (+11/+14)/(+40/+43) (lane 5), Δ(+23/+25) (lane 6), wild-type virus (lane 7), or uninfected Jurkat cells (lane 8). A standard representing an HIV-1 molecular clone present at 0 (lane 9), 10 (lane 10), 10^2 (lane 11), 10^3 (lane 12), or 10^4 copies (lane 13) is also shown. (B) The 110-bp specific band was generated by PCR with a primer pair corresponding to the human β-globin gene to control for DNA content in panel A (lanes 1 to 8).

+159, antisense) that were specific to the R and U5 regions of the HIV-1 LTR. No significant differences were observed in the level of the 139-bp specific band between the chromosomal DNA isolated from Jurkat cells containing the TAR mutant provirusès shown in Fig. 10A for (+19/+22) (lane 1), (+31/+34) (lane 2), (+40/+43) (lane 3), (+11/+14)/(+40/+43) (lane 5), and Δ(+23/+25) (lane 6) compared with the levels for cells containing (+19/+22)/(+40/+43) (lane 4) or the wild type (lane 7). An oligonucleotide primer pair specific to the human β-globin gene, which generated a 110-bp PCR product, was used as an internal control for the quantity of chromosomal DNA in each of the Jurkat cell lines. This primer pair demonstrated no significant differences (Fig. 10B). Thus, the decrease in gene expression in Jurkat cells containing TAR mutant viruses was not due to differences in proviral copy number but, rather, was due to a decreased level of transcription as the result of mutations in TAR.

DISCUSSION

A number of elements in the HIV-1 LTR are critical for the regulation of gene expression. Previous studies have revealed that the enhancer, SP1, TATA, and TAR regions are all critical for both basal and *tat*-induced gene expression (19). Mutations in the enhancer, SP1, and TATA elements have been inserted into HIV-1 proviral constructs and their effects on gene expression and viral growth have been assayed (22, 35, 37, 44, 50). These studies indicated that both the specific regulatory element which was mutated and the cell type which was infected were determinants of the level of viral gene expression. Also, it is important that mutations of some regulatory elements such as the enhancer have effects on viral growth very different from those seen with transient assays. Mutations of NF-κB motifs are very deleterious to HIV-1 gene expression when assayed by transient expression (40), but viruses containing the same mutations exhibit only slight decreases in viral growth properties (35, 50). Thus, it is critical to determine how mutations of different HIV-1 regulatory regions alter gene expression both following transient assays and in viral infection studies. It has not previously been possible to assay the effects of TAR mutations on viral growth and gene expression because of the inability to generate such proviruses. Since TAR is critical for *tat* activation, studies of viruses containing mutations in this regulatory element are critical for a better understanding of the factors controlling HIV-1 gene expression.

Previous mutagenesis studies established that the TAR RNA secondary structure, the primary sequence of the loop, and the bulge element were all critical for *tat*-induced gene expression from the HIV-1 LTR (14, 16, 24, 25, 49, 51, 52, 54). In this study, we used stable 293 cell lines to generate viruses that contained changes in a variety of different portions of TAR. The constitutive expression of the adenovirus E1A protein in 293 cells was able to provide high levels of HIV-1 gene expression, which allowed for the production of these mutant viruses. The use of techniques similar to those described above to generate HIV-1 TAR mutants should allow for the production of other types of retroviruses that contain mutations in critical regulatory elements. The ability to obtain sufficient quantities of such viruses should facilitate the study of regulatory elements which control retroviral gene expression and replication.

T-lymphocyte cell lines infected with either wild-type HIV-1 or the TAR stem restoration mutant virus, (+19/+22)/(+40/+43), resulted in a productive infection, as determined by the levels of secreted p24 Ag and RT activity. However, all other viruses containing TAR mutations, including (+19/+22), (+31/+34), (+40/+43), (+11/+14)/(+40/+43), (+23), Δ(+23/+25), and Δ(+23/+25)/(+37/+39), failed to yield detectable levels of replication in T-cell lines. These results indicate that any mutations that perturb TAR RNA stem structure, the loop, or the bulge are very deleterious for viral gene expression and replication. The defects in gene expression did not appear to be due to differences in the ability of these viruses to infect T lymphocytes or to initiate reverse transcription. Even though most TAR mutant viruses did not give a productive infection in T-lymphocyte cell lines, we were able to use G418 to select Jurkat cells containing roughly equal quantities of each of the TAR mutant proviruses. Though G418-resistant Jurkat cells harboring either the wild type or the TAR stem restoration mutant provirus, (+19/+22)/(+40/+43), expressed similar quantities of p24 Ag, cells containing the TAR mutant proviruses resulted in decreases of viral gene expression ranging from 200- to 5,000-fold compared with that of Jurkat cells harboring the wild-type provirus. These studies of both viral growth and stable gene expression of integrated proviruses agree with previous studies that indicate that TAR is critical for high levels of gene expression from the HIV-1 LTR (4, 14, 16, 24, 25, 49, 52, 54). However, both of these assays demonstrate that the effects of TAR mutations are of much greater magnitude in the context of virus than with plasmid constructs assayed by transient expression.

It is not known if the decreased viral gene expression observed in drug-selected Jurkat cells containing TAR mutant viruses was due to decreased transcriptional initiation and/or decreased elongation. There is considerable experimental evidence that *tat* has only minor effects on promoter-proximal transcription, but it markedly increases promoter-distal transcription (12, 20, 28, 30, 33, 34, 38). Nuclear run-on studies previously performed with HIV-1 proviral constructs which contained a deleted *tat* gene demonstrated that the addition of recombinant *tat* protein to cells containing this HIV-1 proviral construct increased the RNA levels at promoter-distal but not promoter-proximal sites (12). Nuclear run-on experiments with G418-selected Jurkat cells containing TAR mutant proviruses will be required to determine if these viruses are defective in transcriptional initiation or elongation or whether some combination of these effects is present.

While most TAR mutant viruses were unable to efficiently replicate in cultured T-lymphocyte cell lines, differential replication rates were observed when the same viruses were assayed following infection of activated PBMCs. Viruses with mutations that altered the upper portion of the TAR RNA stem structure, (+19/+23) and (+40/+43), replicated more slowly and gave lower p24 Ag levels than viruses containing mutations of the bulge [(+23) and Δ(+23/+25)] or the loop [(+31/+34)]. The ability of some TAR mutant viruses to replicate in activated PBMCs, though at lower rates, was intriguing. However, two mutations in TAR prevented viral replication in activated PBMCs. One mutation, (+11/+14)/(+40/+43), disrupted both the upper and the lower TAR stem structures while the other mutation, Δ(+23/+25)/(+37/+39), transposed the bulge to the opposite side of the TAR RNA stem structure. The latter mutants indicate that the maintenance of the overall structure of TAR RNA is required for the activation of the HIV-1 promoter.

Surprisingly, mutations of the bulge [(+23) and (+23/+25)] or the loop [(+31/+34)] appeared to be less critical for viral replication in PBMCs than mutations that disrupted the upper stem structure [(+19/+22) and (+40/+43)]. Viruses containing mutations in the bulge or the loop exhibited decreases in both viral replication and gene expression, though these de-

fects did not prevent viral growth. These results indicate that *tat* may activate HIV-1 gene expression in the absence of the TAR RNA bulge which is required for Tat binding (4, 6, 8, 10, 11, 51, 52, 58). However, activation of these viruses is not *tat* independent, because mutation of *tat* in these proviral constructs gave no detectable viral gene expression in either 293 cells or T lymphocytes (unpublished observations). In the absence of an intact bulge structure, it is possible that an alternative activation complex which is composed of Tat and cellular proteins binding to the TAR RNA loop can form on TAR. Similarly, it is possible that in viruses with mutations in the TAR RNA loop that Tat bound to the TAR RNA bulge may interact with a complex of cellular proteins that normally bind to the loop. As a test of this model, it will be critical to determine whether viruses containing mutations of both the loop and the bulge are completely defective for replication.

Previous studies using wild-type and TAR mutant proviruses demonstrated that although the wild-type loop sequences and maintenance of TAR secondary structure were required for efficient gene expression in unstimulated Jurkat cells, a TAR-independent gene activation of these proviruses was observed in Jurkat cells treated with phorbol esters (22). Viruses containing mutations of the loop sequence or disruption of the TAR RNA secondary structure expressed near-wild-type levels of p24 Ag in Jurkat cells stimulated with phorbol esters. TAR-independent activation was eliminated by combining mutations of TAR with mutations of the enhancer or by disrupting the *tat* gene (22). The current study revealed that activated PBMCs were more permissive for viral replication of TAR mutants than H9 or Jurkat cells. It seems likely that the ability of TAR mutant viruses to markedly increase their gene expression in stimulated T lymphocytes was achieved by the activation of specific cellular transcription factors. The nature of these cellular factors and the HIV-1 control elements which they regulate to mediate high levels of *tat*-induced gene expression is not known (1). The ability to assay the role of different HIV-1 TAR RNA mutations in the context of virus should help elucidate the RNA elements and mechanisms involved in *tat*-induced gene expression.

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