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Analysis of *trans*-Acting Response Decoy RNA-Mediated Inhibition of Human Immunodeficiency Virus Type 1 Transactivation

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Overexpression of *trans*-acting response element (TAR)-containing sequences (TAR decoys) in CEM SS cells renders cells resistant to human immunodeficiency type 1 (HIV-1) replication. Mutagenesis of TAR was used to investigate the molecular mechanism underlying the observed inhibition. A nucleotide change which disrupts the stem structure of TAR or sequence alterations in the loop abolish the ability of the corresponding TAR decoy RNAs to inhibit HIV replication. A compensatory mutation which restores the stem structure also restores TAR decoy RNA function. Synthesis of viral RNA is drastically reduced in cells expressing a functional TAR decoy RNA, but it is unaffected in cells expressing a mutant form of TAR decoy RNA. It is therefore concluded that overexpression of TAR-containing sequences in CEM SS cells interferes with the process of Tat-mediated transactivation of viral gene expression. However, the phenotype of several mutations suggests that TAR decoy RNA does not inhibit HIV-1 gene expression by simply sequestering Tat but rather does so by sequestering a transactivation protein complex, implying that transactivation requires the cooperative binding of both Tat and a loop-binding cellular factor(s) to TAR. Expression of wild-type or mutant forms of TAR had no discernible effects on cell viability, thus reducing concerns about using TAR decoy RNAs as part of an intracellular immunization protocol for the treatment of AIDS.

The Tat protein of human immunodeficiency virus type 1 (HIV-1) is a potent transactivator of viral gene expression and is essential for viral replication. The *cis*-acting target sequence required for Tat function, the *trans*-acting response element (TAR), coincides with a predicted RNA stem-loop structure present within the first 60 nucleotides of all HIV-1 transcripts (see references 6 and 18 for reviews). Recently we described a strategy to render cells resistant to HIV-1 replication by overexpressing TAR-containing sequences (TAR decoys) in HIV-1-susceptible cells (25). The theory behind this strategy is that since Tat must physically associate with TAR in order to exert its function, overexpression of RNA species encoding TAR could act as decoys for Tat binding and prevent its binding to the TAR sequence present in the viral RNA. Thus, no activation of gene expression and no generation of progeny virus would occur. We have shown that a tRNA-TAR transcription system can be used to express TAR-containing transcripts in CEM SS cells at high levels and render cells highly resistant to HIV-1 replication (25, 26). In this study, we explore the mechanism underlying the observed inhibition by analyzing the effects of sequence alterations on the function of corresponding TAR decoys. The question is whether, as hypothesized, inhibition of HIV-1 in cells expressing TAR decoy RNA occurs by interfering with the process of Tat-mediated transactivation.

Extensive mutagenesis of TAR has revealed that the primary sequence in the loop and the secondary structure of the stem are required for transactivation (2, 8, 10, 13, 14, 20, 22). In addition, the primary sequence of a three-nucleotide bulge in the stem portion of TAR (in particular the invariant U at position 23) is also important for transactivation and is necessary for in vitro binding of purified Tat to TAR (2, 4, 19, 20). Several reports have documented the existence of cellular factors which bind to TAR in vitro (11, 12, 15). Marciniak et al. (15) have identified a 68-kDa cellular factor present in HeLa cell extracts which binds specifically to the loop sequence of TAR. Moreover, mutations in the loop which affected TAR function in vivo also reduced binding of this cellular factor to TAR. The possibility that a cellular factor(s) associates with TAR in vivo raises the concern that overexpression of TAR-containing transcripts in a cell may sequester essential cellular factors and may be deleterious to cell viability or cell function. Although no obvious deleterious effects resulting from the expression of TAR-containing sequences in CEM SS cells were noted (25), the safety of an intracellular immunization approach for the treatment of AIDS based on TAR decoys is brought into question. Therefore, we also wanted to determine whether altered TAR decoy RNAs, which have lost the ability to bind cellular factors, were still capable of inhibiting HIV-1 replication.

MATERIALS AND METHODS

Cells and viruses. The HIV-1 virus strain used in this study was the ARV-2 isolate propagated in HUT 78 cells (21) and was provided by C. Cheng-Mayer and J. A. Levy. CEM SS is a CD4-positive human T-lymphoid cell line that is highly susceptible to infection with HIV-1, including the ARV-2 isolate (17), and was provided by P. L. Nara. CEM SS cells were grown in RPMI supplemented with 10% fetal calf serum (Hyclone).

Construction of retroviral vectors and infection of CEM SS cells. The DC:tTAR retroviral vector was created as previously described (25). The DC:tTARm vectors were created by cloning oligonucleotides encoding the mutant TAR sequences into a DC-type retroviral vector in an analogous manner. Vector DNA was converted to corresponding virus by using established procedures. One microgram of plasmid DNA was electroporated into the amphotropic packaging

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cell line AM12 (16) by using a Bio-Rad gene pulser, and productively transduced cells were selected with G418 (0.7 mg/ml). G418-resistant colonies were pooled, and the vector-containing virus emerging from them was used to infect CEM SS cells. Clonally infected CEM SS cell lines were isolated by G418 selection and limiting dilution in RPMI supplemented with 20% fetal calf serum.

Infection of CEM SS cells with HIV-1. For in situ immunofluorescence and enzyme-linked immunosorbent assay (ELISA) analysis, CEM SS cell lines (2×10^5 cells) were infected with 1 ml of a 1:5 dilution of virus obtained from chronically infected HUT 78 cells in the presence of Polybrene (4 μg/ml) for 2 h. Cells were washed once and resuspended in the original volume. Every 3 days, cells were diluted fivefold in fresh medium. For HIV RNA analysis, 10^6 cells were infected with a 1:5 dilution of virus in 5 ml in the presence of Polybrene.

RNA blot analysis. tRNA blot analysis was performed as previously described (25, 26). To analyze HIV-1 RNAs in infected cells, cytoplasmic RNA was isolated by lysing cells with 50 mM Tris-Cl (pH 8.0)–100 mM NaCl–5 mM $MgCl_2$–0.5% Nonidet P-40 and removing nuclei by centrifugation. The cytoplasmic fraction was then added to 5 ml of guanidium isothiocyanate (3). Total cytoplasmic RNA was isolated by the guanidium-hot phenol method (9). Five micrograms of cytoplasmic RNA was fractionated on a 1% formaldehyde-agarose gel, stained with ethidium bromide to ascertain that equal amounts of RNA were loaded in each lane, transferred to a nylon membrane (Biotrans; ICN) by using a Bio-Rad electroblotter, and UV cross-linked to the membrane with a Stratagene UV Stratalinker. The membrane was prehybridized at 65°C for 5 min in 25 ml of 1% bovine serum albumin–1 mM EDTA–0.5 M Na_2PO_4 (pH 7.2)–7% sodium dodecyl sulfate. A ^{32}P-labeled *tat* gene probe (10^7 cpm) was generated with an oligolabeling kit (Stratagene) and added directly to the prehybridization solution. Hybridization was carried out overnight at 65°C. Washed membranes were exposed to X-ray film (XAR5) at −70°C for 4 days.

In situ immunofluorescence and p24 ELISA analysis. CEM SS cells (10^5) were washed once with phosphate-buffered saline (PBS), applied to microscope slides, and fixed with cold methanol-acetone (2:1). Cells were incubated with human anti-HIV serum diluted 1:80 for 1 h at 37°C, washed with PBS, and incubated with fluorescein isothiocyanate-conjugated goat anti-human antibody (Cappel) diluted 1:50. Cells were washed with PBS and examined under a fluorescence microscope. The abundance of the HIV p24 protein in day 17 postinfection supernatants was determined by using a p24 antigen-specific ELISA kit (no. NEK-060) from Dupont.

RESULTS

Three mutant TAR decoy RNAs were analyzed in these studies. As shown in Fig. 1A, TARm-1 contains a G nucleotide at position +29 (G-29) instead of C, which disrupts the stem structure at the base of the loop. This mutation was shown to abolish transactivation (8) and to reduce the in vitro binding of purified Tat fourfold (27) as well as reduce the binding of the HeLa cell-derived cellular factor twofold (15). A similar alteration in TAR in which G-36 is changed to C reduced Tat binding less than twofold (7). TARm-2 contains two alterations: C-29 to G, as in TARm-1, and G-36 to C. The second alteration restores the secondary

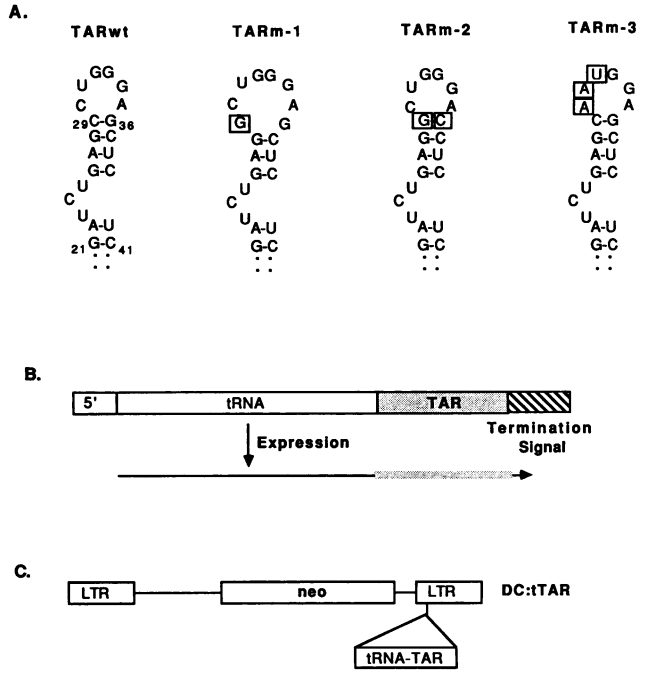

FIG. 1. Structure of chimeric tRNA-TAR DNA templates and retroviral vectors. Chemically synthesized oligonucleotides corresponding to the first 60 nucleotides of the HIV-1 (ARV-2 strain) RNA and three derivatives, TARwt and TARm-1 to TARm-3, respectively (A), were fused to the 3′ end of a human $tRNA_i^{Met}$ derivative to yield a fusion RNA transcript (B). The chimeric tRNA-TAR DNA templates were inserted into the 3′ LTR of the murine retroviral vector N2A. (For additional details, see Materials and Methods and references 25 and 26.) Nucleotide alterations in the TAR sequence from the wild-type sequence are indicated by boxes.

structure of TAR which was disrupted in TARm-1. Feng and Holland (8) have shown that such a compensatory mutation restores the ability of the mutant TAR to function in a transactivation assay, and Marciniak et al. (15) have shown that binding of a cellular factor is restored to wild-type levels. TARm-3 contains three alterations in the loop. Sequence alterations in the loop were shown to be essential for transactivation (2, 8, 10, 20, 22) and for binding of the HeLa cell-derived cellular factor (15) but had no effect on Tat binding (4, 19).

CEM SS-derived cell lines expressing wild-type and mutant TAR decoy RNAs were generated essentially as previously described (25) (see also Materials and Methods). Briefly, chemically synthesized oligonucleotides corresponding to wild-type TAR and the three mutant TARs shown in Fig. 1A were fused to a human $tRNA_i^{Met}$ gene (Fig. 1B) and were inserted into the 3′ long terminal repeat (LTR) of N2A, a Moloney murine leukemia-derived retroviral vector (Fig. 1C). The resulting vector constructs were converted to corresponding virus and used to infect CEM SS cells. CEM SS is a human $CD4^+$ T-cell line which is highly susceptible to infection with HIV-1 (17). Clonal isolates of CEM SS cells harboring a single provirus were isolated by limiting dilution in the presence of G418 and used in subsequent studies. To determine whether clonal CEM SS cell lines transduced with DC:tTAR and DC:tTARm vectors express comparable amounts of chimeric tRNA-TAR transcripts, total RNA was isolated, subjected to electrophoresis

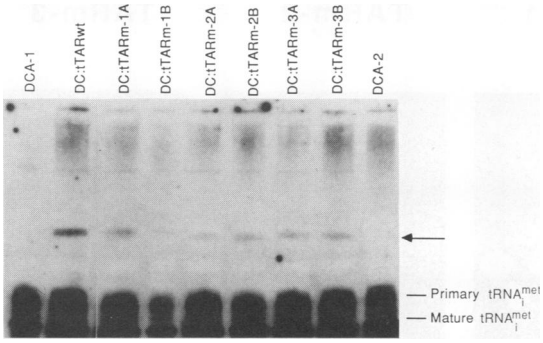

FIG. 2. RNA blot analysis of tRNA-TAR transcripts expressed in CEM SS cells. Total RNA was isolated by the guanidium isothiocyanate method (9) from clonally derived CEM SS cell lines harboring the DC:tTARwt vector, two independent clones of DC:tTARm-1 to -3 (clones A and B), and two clones harboring the unrelated vector DCA (clones 1 and 2). Twenty micrograms of total RNA was fractionated on an 8% polyacrylamide-urea gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled human tRNA$_i$ Met probe. The band corresponding to the 147-nucleotide-long tRNA-TAR transcripts is indicated by the arrow. The bands corresponding to the endogenous 89- and 72-nucleotide-long transcripts of the primary and mature tRNA$_i$ Met transcripts are also indicated.

in an 8% polyacrylamide-urea gel, transferred to nylon paper, and hybridized with a tRNA$_i^{Met}$-specific probe. As shown in Fig. 2, comparable amounts of tRNA-TAR fusion transcripts are synthesized in cells harboring the wild-type TAR and mutant TAR templates. The small variation in the intensity of the bands corresponding to the chimeric tRNA-TAR transcripts (indicated by the arrow in Fig. 2) is primarily due to variations in the amount of RNA loaded per lane, as indicated by similar variations in the intensity of bands corresponding to the endogenous tRNA$_i^{Met}$ transcripts.

Expression of tRNA-TARwt transcripts in CEM SS cells had no discernible effects on cell viability. Figure 3 shows that expression of tRNA-TARwt or tRNA-TARm-3 transcripts in CEM SS cells had no effect on their growth rate over an extended period of time. Furthermore, microscopic examination revealed no morphological differences between CEM SS cells and clonal isolates expressing tRNA-TAR transcripts (data not shown).

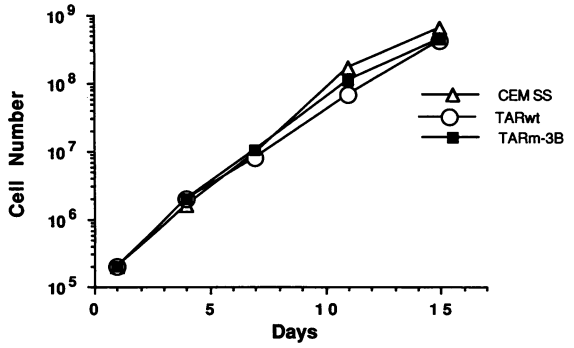

FIG. 3. Rate of proliferation of CEM SS cells expressing tRNA-TAR transcripts. Two clonal isolates of CEM SS cells shown in Fig. 2, DC:tTARwt and DC:tTARm-3B, as well as parental CEM SS cells were seeded at 2×10^5 cells per ml. Every 3 or 4 days, the total cell number was determined and the cell culture was diluted fourfold with fresh medium.

CEM SS cell lines expressing tRNA-TARwt or tRNA-TARm transcripts were analyzed for their ability to support the replication of HIV-1. Cells were infected at low multiplicity, and 17 days postinfection, the extent of virus spread through the culture was determined by in situ indirect immunofluorescence and by using an ELISA test for the detection of the p24^{gag} antigen in the cell supernatant. Figure 4 shows that both a single-nucleotide alteration which disrupts the stem structure of TAR (TARm-1) or changes in the nucleotide sequence of the loop (TARm-3) abolish the ability of the corresponding TAR decoy RNAs to protect cells from HIV-1 replication. However, a compensatory mutation which restores the integrity of the stem (TARm-2) restores the ability of the corresponding decoy RNA to protect cells from HIV-1 replication.

If TAR decoy RNAs inhibit HIV replication by interfering with Tat-mediated transactivation, an overall decrease in viral RNA synthesis should be observed in cells expressing a wild-type form but not in cells expressing a mutant form of TAR decoy RNA. Viral RNA accumulation was analyzed in cells 5 days postinfection with HIV-1. As shown in Fig. 5, accumulation of viral RNA in the cytoplasm is much lower in cells expressing TARwt decoy RNA than in parental CEM SS cells or cells harboring a biologically inactive mutant form of TAR decoy RNA, TARm-3. Analysis of viral RNA in cells 3 days postinfection with HIV-1 yielded similar results (data not shown). At this time point of infection, minimal spread of virus has taken place, as suggested by analysis of proviral DNA content (data not shown) and in situ indirect immunofluorescence of HIV-infected cells (25).

DISCUSSION

The main conclusion from this analysis is that sequence alterations in TAR which affect its in vivo function, as measured in various transactivation assays, have a similar effect on the ability of TAR decoy RNAs to inhibit HIV-1 replication in human CD4 T cells. This correlation strongly suggests that overexpression of TAR-containing sequences in human CD4 T cells inhibits HIV-1 replication by interfering with the process of Tat-mediated transactivation of viral gene expression and not by some nonspecific antiviral effect. Furthermore, it provides direct evidence that Tat-mediated transactivation, as measured in various transactivation assays, also occurs in virus-infected cells. Use of TAR decoy RNAs represents a novel and complementary approach to study the mechanism of Tat-mediated transactivation. The two main advantages of this approach, which is essentially an in vivo competition study, are that the experiments are performed with human CD4 T cells and HIV-1 replication is measured, whereas other transactivation assays measure transient expression of TAR-containing DNA constructs, often in heterologous cell types.

A rather unexpected finding was that expression of TARm-3 decoy RNA, which contains an altered loop sequence, did not inhibit HIV-1 replication. One possible interpretation of this observation is that TAR decoy RNA does not function by sequestering Tat but rather prevents transactivation by sequestering the loop-binding cellular factor (15). An alternative possibility is that Tat does not bind to TARm-3 RNA in vivo because binding of Tat to wild-type TAR in vivo is dependent on the interaction of

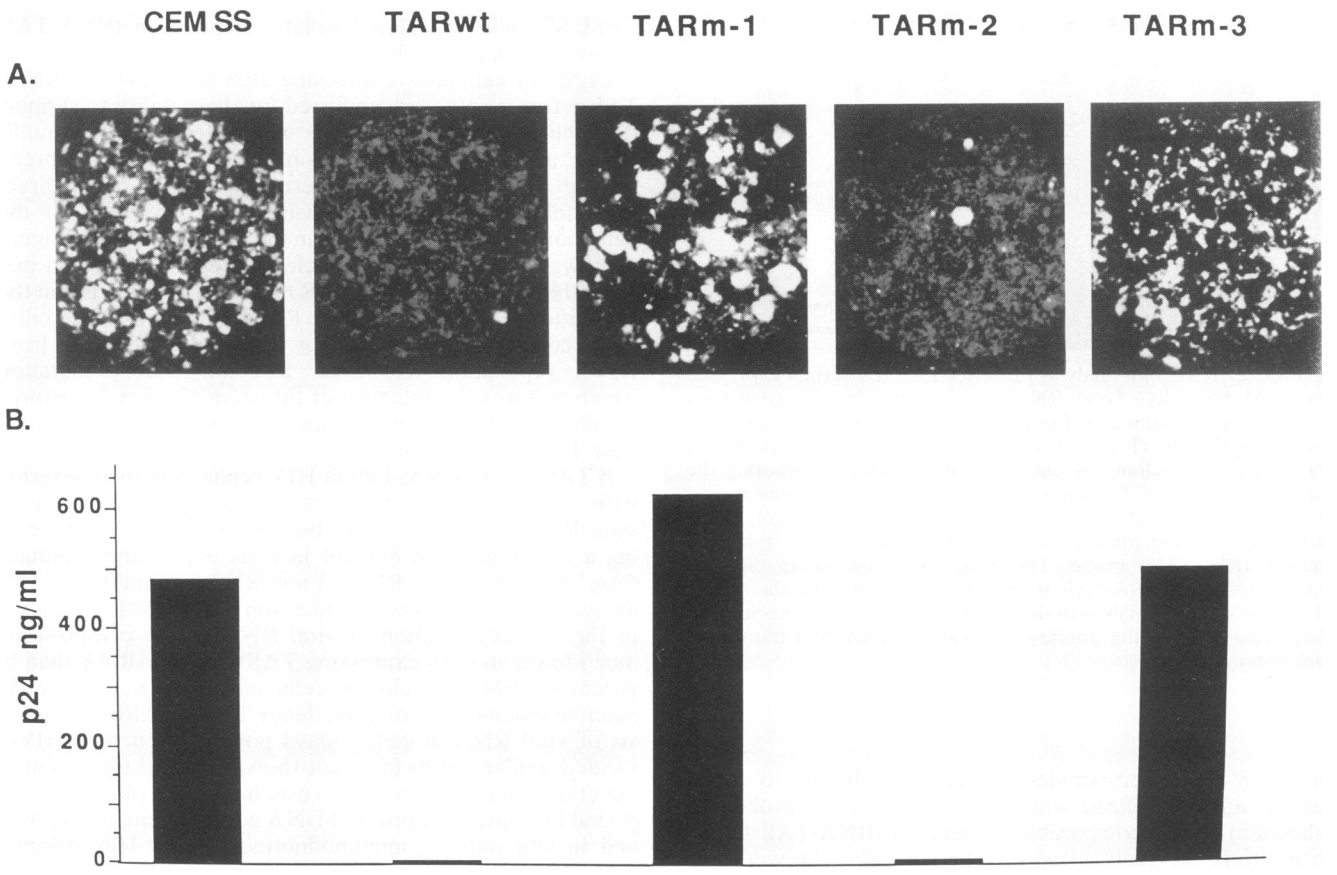

FIG. 4. HIV-1 replication in CEM SS cells expressing wild-type and mutant forms of TAR decoy RNA. Cell lines expressing either the wild-type TAR decoy, mutant TAR decoy, or parental CEM SS cells were infected with HIV-1 virus (ARV-2 strain [21]); 17 days postinfection, spread of virus was determined by indirect in situ immunofluorescence (A) and by secretion of progeny virus into the culture medium as measured by a p24 ELISA (B). Two cell lines were analyzed in each group. A representative field from one cell line from each group is shown in panel A. p24 values shown in panel B are averages obtained from both cell lines.

TAR with the loop-binding cellular factor. The observation that TARm-1 does not inhibit HIV argues against the first possibility. If TAR decoys were to inhibit HIV replication by sequestering the loop-binding cellular factor, then TARm-1 should be an effective decoy because the mutation in TARm-1 RNA causes only a twofold reduction in the affinity of the cellular factor to TAR (15).

Studies involving the use of chimeric proteins consisting of the transactivation domain of Tat fused to heterologous RNA- or DNA-binding domains have shown that Tat is the direct and sole mediator of the transactivation signal and that the only function of TAR is to position Tat in close proximity to its site of action (1, 23, 24). Other studies have shown that alterations in the primary sequence of the loop abolish or reduce transactivation but have no effect on the in vitro binding of Tat to TAR (2, 4, 8, 10, 19, 20, 22). The latter studies suggest that additional components, such as the recently identified loop-binding cellular factor (15), are required for transactivation. This apparent contradiction can be reconciled if, as our studies suggest, the function of the loop-binding cellular factor is to stabilize the interaction between Tat and TAR. Cooperativity between Tat and the HeLa cell-derived cellular factor for binding to TAR was suggested by Cullen (5).

The cooperativity of binding between Tat and the loop-binding cellular factor may be reciprocal; i.e., the binding of the cellular factor may itself be dependent on the binding of Tat. Overexpression of TAR-containing sequences in cells would have been expected to sequester any cellular factor which binds to the TAR sequences. Nevertheless, no deleterious effect on the viability of CEM SS cells expressing TAR decoy RNA was noted regardless of its ability to bind the cellular factor (25) (Fig. 3). It is possible that such factors are present in excess in CEM SS cells or that they are dispensable for cell viability. It is, however, tempting to speculate that the reason that expression of TAR decoy RNA is not deleterious to the cell is that it does not bind cellular factors in the absence of Tat. If this is the case, a serious concern about the safety of a TAR decoy-based inhibition strategy for the treatment of AIDS will have been eliminated.

In summary, mutations in the stem or loop structure of TAR have similar effects on the ability of TAR decoy RNA to inhibit HIV replication in CEM SS and on Tat-mediated transactivation of the HIV LTR-linked reporter genes as measured in transient transactivation assays. However, sequence alterations in the loop of TAR which have no effect on binding of Tat to TAR in vitro render the corresponding TAR decoy RNA (TARm-3) nonfunctional. This finding suggests that TAR decoy RNA does not exert its function by simply sequestering Tat but rather does so by sequestering a transactivation protein complex and that binding of Tat to

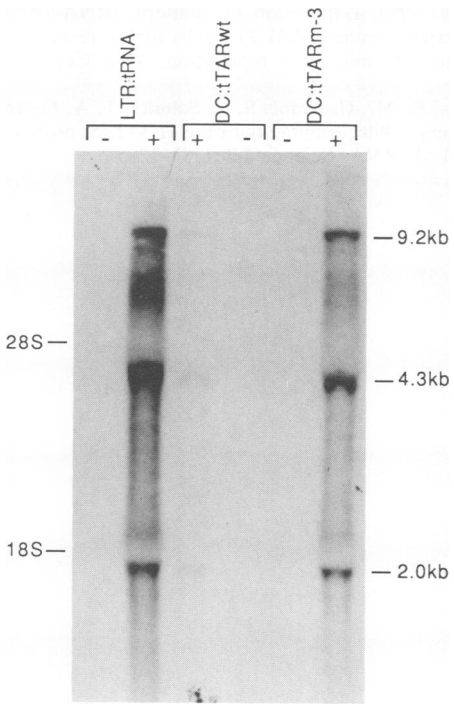

FIG. 5. RNA blot analysis of HIV-1-infected CEM SS cells. Five days postinfection, total cytoplasmic RNA was isolated from mock-infected (−) and HIV-1-infected (+) CEM SS cells harboring an LTR:tRNA, DC:tTARwt, or DC:tTARm-3 retroviral vector. (The LTR:tRNA vector described in references 26 is similar to the DC:tTARwt vector except that it lacks the TAR sequence.) HIV-1-specific RNA transcripts were detected with a *tat*-specific probe. The three major bands detected correspond to the viral unspliced (9.3-kb), singly spliced (4.2-kb), and multiply spliced (2.0-kb) RNA species.

TAR in vivo requires the binding of additional cellular factors to TAR.

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