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Tat-responsive region RNA of human immunodeficiency virus type 1 stimulates protein synthesis *in vivo* and *in vitro*: Relationship between structure and function

(translational control/interferon/eukaryotic initiation factor 2 kinase/phosphorylation)

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ABSTRACT **The Tat-responsive region (TAR) sequence is present at the 5′ end of human immunodeficiency virus 1 mRNAs and as a cytoplasmic form of 58–66 nucleotides. TAR RNA blocks the activation and autophosphorylation of the double-stranded RNA-activated protein kinase *in vitro*. We show here that TAR RNA also prevents the double-stranded RNA-mediated inhibition of translation in a cell-free system. Mutagenic and structural analyses of TAR RNA indicate that a stem of at least 14 base pairs is required for this activity, whereas the loop and bulge required for transactivation by Tat are dispensable. Truncation of the RNA to 68 nucleotides results in the loss of translational rescue ability, suggesting that the short cytoplasmic TAR RNA produced by viral transcription *in vivo* may not have the capability to suppress activation of the kinase. However, because longer TAR transcripts stimulate expression in a transient assay *in vivo*, the TAR structure at the 5′ end of viral mRNAs could still exert this function *in cis*.**

Human immunodeficiency virus type 1 (HIV-1) encodes several regulatory proteins essential for its replication (1, 2). Tat is a powerful transactivator of viral gene expression that acts through an RNA element, the Tat-responsive region (TAR) located between nucleotides (nt) +14 to +44, relative to the transcriptional start site in the long terminal repeat. TAR RNA forms a stable structure containing a stem, bulge, and loop, in which the integrity of the bulge and adjacent stem is important for interaction with the Tat protein. Tat interacts directly with TAR RNA to stimulate viral gene expression at the transcriptional level and possibly at posttranscriptional levels (1, 2). TAR RNA is present at the 5′ end of HIV-1 primary transcripts and mRNAs and as a cytoplasmic form of 58–66 nt (3, 4). The stability of this short form (3–8) suggests that it may play a role in the viral life cycle, and its apparently cytoplasmic location has prompted investigations of a translational function (9–14).

TAR RNA interacts with several cellular proteins, including a protein kinase, the double-stranded (ds)RNA-activated inhibitor of translation (DAI) (10–14). Synthesis of this enzyme (also referred to as p68 kinase, dsI, and P1) is induced by interferon, and it is important in the cellular defense against viral infection (15). DAI occurs in an inactive state in most mammalian cells and is activated during viral infection by dsRNA-mediated autophosphorylation. The activated enzyme phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2) (16), which then traps another initiation factor, the guanine nucleotide exchange factor (GEF or eIF-2B). Protein synthesis is thereby blocked, and viral propagation is impaired.

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Several viruses have developed ways to combat the DAI-mediated cellular defense mechanism (17). Particularly relevant here are the adenovirus virus-associated (VA) RNAs and Epstein–Barr virus-encoded RNAs (EBERS), small highly structured RNAs that block the activation of DAI by dsRNA (18). Similarly, the TAR RNA of HIV-1 can inhibit the activation of DAI by dsRNA in a kinase assay containing purified enzyme and RNA (13). We have extended this observation to more physiological systems, both *in vivo* and *in vitro*. The rabbit reticulocyte lysate responds to a variety of physiological stimuli that regulate translation and in many ways affords a reliable representation of the *in vivo* situation (19). TAR RNA blocks dsRNA-mediated inhibition of cell-free translation, indicating that it inhibits activation of DAI by dsRNA in this system. To determine the structural features of the RNA that are important for its DAI inhibitory property, we made mutations at different positions in the TAR sequence. The mutant RNAs were examined structurally and tested for their translational rescue ability in cell-free translation. The results show that the stem of the molecule, but not its Tat-binding elements, is crucial for activity and imply that the short cytoplasmic form of TAR RNA may not be able to act as a translational activator *in vivo*. However, protein synthesis *in vivo* was stimulated by longer versions of TAR RNA expressed from a newly constructed vector. These observations suggest that TAR RNA may prevent DAI activation in an intact cell when located at the 5′ end of viral mRNAs.

RESULTS

TAR RNA Suppresses the dsRNA-Mediated Inhibition of Translation. Experiments conducted *in vitro* with purified components suggested that TAR RNA might prevent the inhibition of protein synthesis that results from activation of DAI by dsRNA (13). To extend these results to a more physiological setting, we examined the effect of TAR RNA on globin synthesis in the rabbit reticulocyte lysate translation system. In this system DAI is ribosome-bound (19) and probably in a more native state than the purified enzyme used in the kinase assay. The results of experiments designed to test the ability of this RNA to inhibit translation and to prevent translational inhibition by dsRNA are shown in Fig. 1 *A* and *B*, respectively.

TAR RNA, purified to remove traces of dsRNA contaminants, does not activate DAI (13). To verify that similarly purified TAR RNA is not inhibitory to cell-free translation, we preincubated the RNA with the reticulocyte lysate to

Abbreviations: HIV-1, human immunodeficiency virus type 1; TAR, Tat-responsive region; nt, nucleotide(s); DAI, the double-stranded RNA-activated inhibitor of translation; ds, double-stranded; eIF-2, eukaryotic initiation factor 2; RNA_I, inhibitory RNA; CAT, chloramphenicol acetyltransferase; VA, virus associated.

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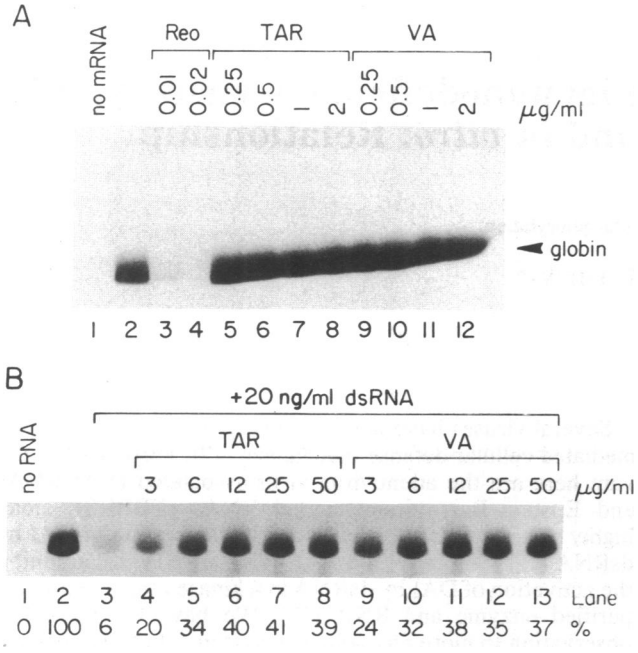

FIG. 1. Effect of wild-type TAR RNA on translation. (*A*) Neither TAR RNA nor adenovirus VA RNA inhibits translation *in vitro*. Rabbit reticulocyte lysate was preincubated for 15 min at 30°C with water (lanes 1, 2), reovirus dsRNA (Reo; lanes 3, 4), TAR RNA (lanes 5–8), or VA RNA_I (lanes 9–12). The reaction mixture was completed by adding globin mRNA (5 μg/ml; Bethesda Research Laboratories), [^{35}S]methionine [5 mCi/ml (1 Ci = 37 GBq); ICN], 0.4 mM GTP, and other components as specified (20) and incubated for a further 30 min at 30°C. Translation products were resolved in a 15% polyacrylamide/SDS gel and visualized by fluorography. No mRNA was added to the reaction analyzed in lane 1. The globin band is marked. (*B*) TAR RNA and VA RNA reverse inhibition by dsRNA. Reticulocyte lysate was preincubated with reovirus dsRNA alone (lane 3), or with TAR RNA (lanes 4–8), or VA RNA_I (lanes 9–13) and then assayed for its ability to translate globin mRNA. Control reactions contained no RNA (lane 1) or globin mRNA only (lane 2). The globin band was quantified by using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager, and relative incorporation is indicated.

permit activation of DAI. The remaining components were added to complete the translation reaction, and globin synthesis was monitored. Preincubation with reovirus dsRNA reduced translation to ≈6% of the control value (Fig. 1*A*, compare lanes 3 and 4 with lane 2), presumably by activating DAI and causing eIF-2α phosphorylation. Preincubation with TAR RNA had no effect on translation over a range of concentrations (lanes 5–8), indicating that it fails to activate DAI. Similarly, VA RNA_I also had no effect on translation (lanes 9–12). These observations are consistent with conclusions drawn from kinase assays by using purified DAI (13, 21).

TAR RNA, like VA RNA (18), blocks the autophosphorylation of DAI by dsRNA in a direct kinase assay (13). To examine this property in the cell-free translation system, we tested the ability of TAR RNA to reverse the dsRNA-mediated inhibition of globin synthesis in the rabbit reticulocyte lysate. The lysate was preincubated with reovirus dsRNA together with TAR RNA or VA RNA; then the remaining components were added, and translational capability was assayed by measuring globin synthesis as above. Inclusion of TAR RNA in the preincubation rescued translation to ≈40% of the control level (Fig. 1*B*, lanes 4–8). Similar restoration of translational capacity was obtained with adenovirus VA RNA (lanes 9–13) and with high concentrations of dsRNA (>1 μg/ml), neither of which completely prevents DAI activation in a kinase assay (data not shown). It is unclear whether the incompleteness of the rescue is due to the preincubation protocol or to the existence of other mechanisms of inhibition by dsRNA besides DAI activation in the reticulocyte lysate translation system. Nevertheless, these data demonstrate the ability of TAR RNA and VA RNA to inhibit DAI activation in a cell-free translation system.

Structural Requirements for TAR RNA Activity. DAI interacts with dsRNA and structured single-stranded RNA molecules. To define TAR RNA features that are required for its translational role, we made a variety of mutations to change its structure (Fig. 2*A*). All of these variant TAR RNAs were tested in the translation assay (Fig. 3) and were subjected to secondary-structure analysis (Fig. 4). As illustrated in Fig. 4*A*, the S1 and BS mutants are deleted in the apical loop and at the top of the stem; mutants 3R (9) and NF have a substitution and an insertion in the stem region, respectively; the AfI and Nhe truncations shorten the RNA to 68 and 48 nt, respectively; and in RAT RNA the sequence of nt 3–57 is reversed, whereas that of nt 58–82 is present in the normal orientation.

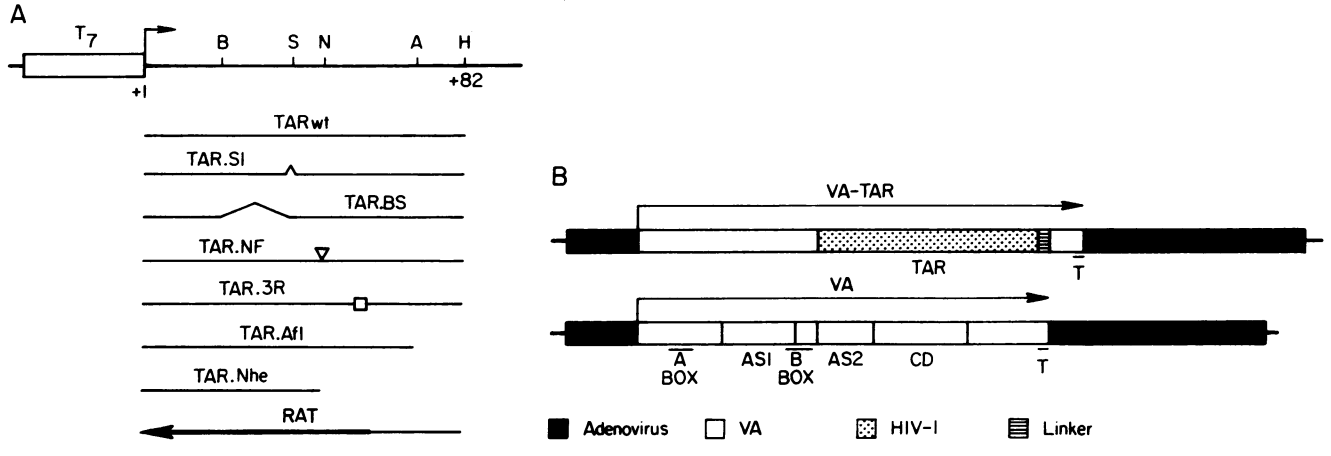

FIG. 2. TAR expression constructs. (*A*) T7 expression plasmids based on pEM-7 were used to transcribe TAR RNA *in vitro* (13). RAT construct contains nt 3–57 of TAR in reverse orientation. Transcription was run off at the *Hin*dIII site (H) at +82 of the TAR sequence or at the *Afl* I(A) or *Nhe* I(N) sites. Restriction sites used to make mutations are also marked (B, *Bgl* II; S, *Sac* I). (*B*) pVA (*Lower*) contains the adenovirus 2 VA RNA_I gene (22) cloned into pUC119. In the pVA-TAR (*Upper*), TAR sequences are substituted for a region in the 3′ half of the VA RNA_I gene (*Lower*). Marked are the A box and B box of the VA RNA promoter, the termination sequence (T), and the regions encoding the apical stem (AS1 and AS2) and central domain (CD) of VA RNA.

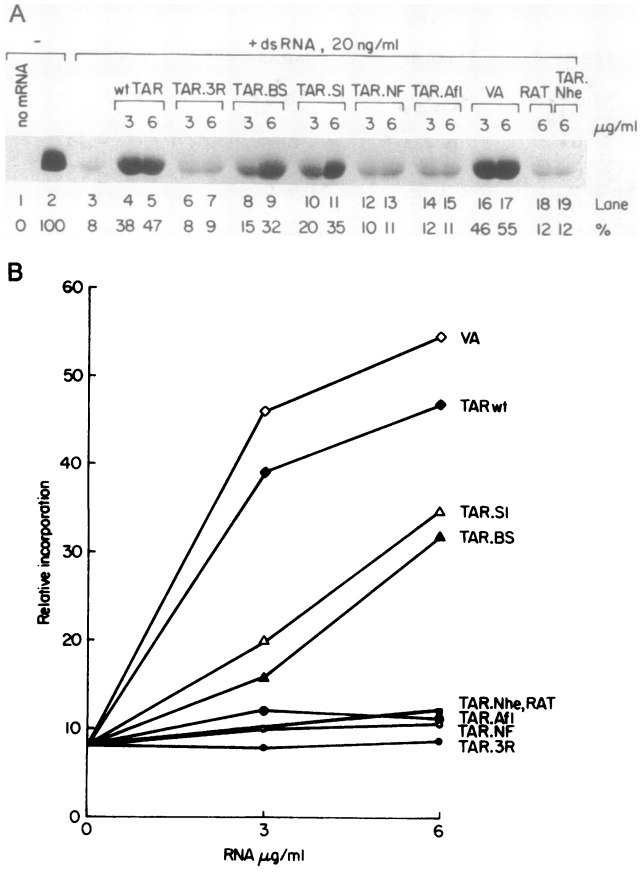

FIG. 3. Rescue of translational inhibition by TAR RNA mutants. Rabbit reticulocyte lysate was preincubated with reovirus dsRNA alone (lane 3) or with wild-type (wt) TAR RNA (lanes 4, 5), mutant TAR RNAs (lanes 6–15, 18, 19), or VA RNA_I (lanes 16, 17) and then tested for its capability to translate globin mRNA. Control reactions contained no RNA (lane 1) or globin mRNA only (lane 2). Globin synthesis was analyzed by gel electrophoresis (*A*) and quantified (*B*).

Alone, none of the RNAs inhibited protein synthesis, demonstrating that they were free of dsRNA contamination (data not shown). In the presence of dsRNA, only mutants S1 and BS were able to rescue translation. They were ≈60% as active as wild-type TAR RNA, whereas the remaining mutants, NF, 3R, Afl, Nhe, and RAT, were essentially inactive (Fig. 3). The results suggested that the stem and terminal stem–loop structures are essential for DAI interaction, whereas the apical loop is dispensable. The ineffectiveness of the RAT RNA suggested that the sequence of the stem might also be important because this RNA was expected to form a structure that is the mirror-image of the TAR stem–loop. Before drawing these conclusions, however, it was necessary to examine the structures of the variant RNAs experimentally because it is difficult to predict RNA secondary structures from primary sequence.

To this end we used the technique of nuclease-sensitivity analysis. The mutant TAR RNAs were probed with a battery of structure-sensitive nucleases, and the cleavage sites were determined. Secondary-structure models were derived by use of a computer program that calculates the most stable structure based on thermodynamic parameters (23), modified to incorporate the empirical data defining unpaired nucleotides. The model for wild-type TAR RNA built using this procedure closely resembles the structure predicted from sequence alone (24, 25), except that the bulge contains 4 nt instead of 3 nt, in agreement with chemical-modification data (26). For the mutant RNAs, however, the discrepancies were wider because the stability of short duplexes was exaggerated in the absence of empirical data.

The two functional mutants, S1 and BS, formed simple stem–loops. They retained a substantial part of the wild-type stem, 20 and 14 base pairs (bp), respectively. In both cases the deletion caused the formation of a larger loop differing in sequence from that in wild-type TAR RNA. The 3′ terminal stem–loop structure was retained in mutant S1, but in mutant BS this sequence was essentially single stranded, suggesting that this structure is influenced by tertiary interactions with the rest of the molecule. We infer that a stem structure is important for function and that 14 bp is sufficient. The sequence of the apical loop is not critical, and neither the bulge nor the 3′ terminal stem–loop is necessary for function.

The stem mutants 3R and NF exhibited disrupted stems, as expected, but maintained the wild-type apical loop. Mutant NF retained the wild-type 3′ terminal stem–loop structure, whereas mutant 3R adopted a different structure, again consistent with an interaction of this region with the rest of the molecule. These mutants were inactive (Fig. 3), consistent with the deduction that the stem is important for the DAI inhibition property and that the bulge–loop at the top of the stem and the 3′ terminal stem–loop structure are dispensable for this function.

The two truncations, Afl and Nhe, were also inactive. Nhe is essentially single-stranded, which accounts for its behavior. Afl resembles wild-type TAR RNA, except that the 3′ terminal stem–loop structure is amputated and the RNA exhibits increased sensitivity to single-strand specific nucleases indicative of structural instability in the stem region. It is not clear whether this instability or the foreshortening of the 3′ tail is responsible for the loss of function, but with the caveat that *in vivo* conditions might be different, it appears that TAR transcripts of the size seen in transfected cells are unable to block DAI activation.

As a result of the reversal of the sequence between nt 3 and 57 in RAT RNA, the stem, bulge, and loop structure was expected to remain intact but in an opposite orientation. Unexpectedly this RNA displayed a very open structure, lacking any duplex longer than 4 bp, although the 3′ terminal stem–loop structure of the molecule retained the wild-type structure. The inactivity of RAT RNA in the translation assay (Fig. 3) is again consistent with the inference that inhibition of DAI activation requires the TAR stem structure.

TAR Containing RNA Stimulates Expression from a Transfected Plasmid. These results confirmed that purified TAR RNA can prevent activation of DAI by dsRNA *in vitro* in a reticulocyte lysate translation assay as well as in a kinase assay and identified the structural element required. To determine whether the same response is obtained *in vivo* and without RNA purification, we used a transient expression assay developed to study the function of VA RNA (27–30). Transfection of the plasmid pβCAT, containing the chloramphenicol acetyltransferase (CAT) gene under the direction of the mouse β-globin promoter (28) into 293 cells, leads to activation of DAI and eIF-2 phosphorylation (27). The resultant poor expression of CAT is greatly stimulated by cotransfection of a plasmid producing the VA RNA that blocks activation of DAI (27, 28).

To express TAR RNA *in vivo*, we constructed the pVA-TAR plasmid, in which TAR is placed downstream of the strong VA RNA_I gene promoter (Fig. 2*B*). Because this RNA polymerase III promoter is intragenic, the transcribed RNA is chimeric: VA–TAR RNA is ≈170 nt long, containing the TAR sequence (−3 to +82) flanked by 68 nt of VA RNA sequence on its 5′ side and 9 nt on its 3′ end. The residual adenovirus sequences lack both the VA RNA features required for blocking DAI activation, the central domain and the apical stem (Fig. 2*B*). When examined by nuclease-sensitivity analysis, the regions derived from VA RNA were

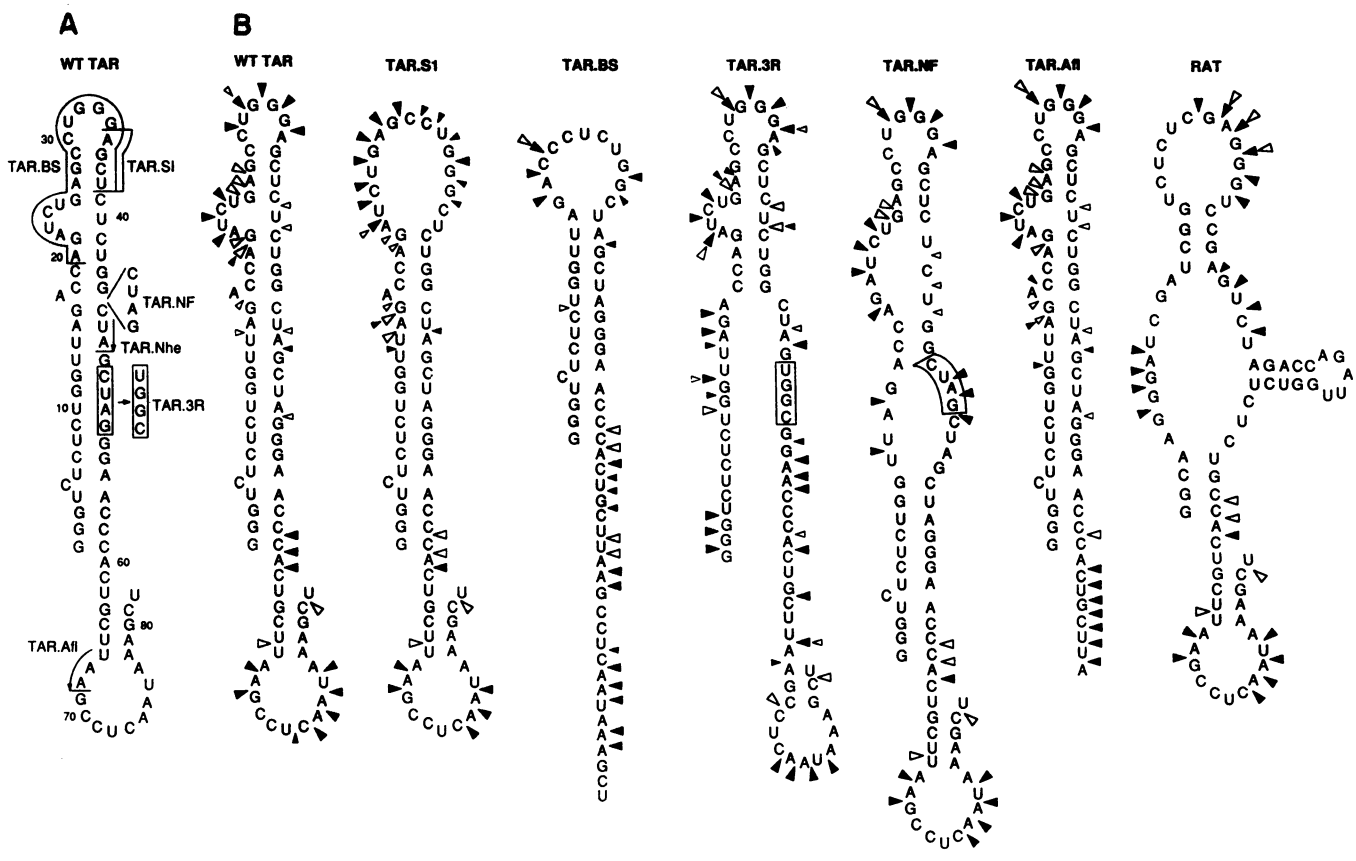

FIG. 4. Structure of TAR RNA mutants. (*A*) Locations of TAR mutations are indicated on wild-type (WT) TAR RNA structure. (*B*) Secondary structures of wild-type and mutant TAR RNAs were determined by the nuclease-sensitivity method (22). 5'-End-labeled RNA was partially digested with RNase T_1, U_2, *Bacillus cereus*, or T_2 (cleaving single-stranded RNA at guanine, adenine, pyrimidines, or any base, respectively), or by cobra venom RNase V_1 (specific for duplex regions or stacked nucleotides), and analyzed in denaturing polyacrylamide gels. Solid arrowheads indicate positions of single-strand specific cuts; open arrowheads indicate nuclease V1 cuts. Size of the arrowhead denotes cleavage intensity.

unstructured, as evidenced by frequent single-stranded cuts (Fig. 5*C*). However, the TAR RNA structure in VA–TAR was essentially identical to that in free TAR RNA. Thus, the main structure observed in the molecule was the stem and loop structure of TAR RNA together with a small 3' terminal stem–loop.

Cotransfection of pVA-TAR with the pβCAT reporter plasmid resulted in an 8-fold stimulation of CAT enzyme activity (Fig. 5*A*). In several experiments, the stimulation ranged from 3- to 9-fold. In a parallel assay VA RNA stimulated by 34-fold (range 6- to 34-fold). RNA blot analysis showed that VA RNA accumulated three to five times the level of VA–TAR RNA (Fig. 5*B*), which presumably accounts, at least in part, for its lesser ability to stimulate expression of the reporter plasmid.

DISCUSSION

The Tat protein and TAR element exert a profound effect on HIV-1 gene expression, and evidence has been adduced for actions at several levels including translation. Here we have addressed the interaction between the TAR transcript and the cellular protein kinase DAI, a key component of the cellular antiviral defense mechanism triggered by interferon. Based on studies in model systems, both positive and negative actions have been reported. Initial work suggested that TAR RNA activates DAI *in vitro*, thereby blocking viral gene expression (10–12, 14). To obtain synthesis of viral proteins, it was proposed that the inhibitory effect of TAR RNA is overcome either directly (12) or indirectly (35–37) by Tat. By contrast, our earlier work indicated that TAR RNA purified to remove dsRNA contaminants acts as a positive effector, preventing the activation of DAI by dsRNA (13). Like small structured RNAs encoded by other viruses (18), this HIV-1 RNA would forestall the inhibition of protein synthesis initiation that limits translation and viral multiplication. The present data demonstrate that TAR RNA can prevent the inhibition of protein synthesis both *in vivo* and *in vitro* and argue strongly that this ability to block DAI activation is a property of the enzyme and RNA in their native states.

These findings establish the potential of TAR RNA to counteract the interferon-induced defense mechanism *in vivo*, but further information is required to show that it fulfills this role in the infected cell. As pointed out (13), the structure of TAR RNA is compatible with its ability to block DAI activation by dsRNA but not to activate DAI on its own. Activation requires a perfectly matched RNA duplex of at least 30 bp, not present in TAR RNA, whereas shorter duplex regions such as those in TAR RNA can bind to the enzyme and prevent its activation (38, 39). Analysis of deletion mutants of TAR RNA led to the conclusion that a duplex of 14 bp is necessary for the DAI-inhibitory function of TAR RNA, consistent with the observation that the stem region of TAR RNA is required for DAI binding (14). However, the inactivity of the TAR.AfI truncation suggests that this length of duplex may not be sufficient for DAI inhibition. Although the presence of the 3' stem and loop structure seen in the 82-nt-long TAR RNA does not correlate with function, its removal results in loss of function, suggesting that a single-stranded tail of >8 nt is required. Taken at face value, this observation would militate against the hypothesis that the 58- to 66-nt TAR transcripts found *in vivo* (8) play a role in blocking DAI activation in the cell. Moreover, it is doubtful

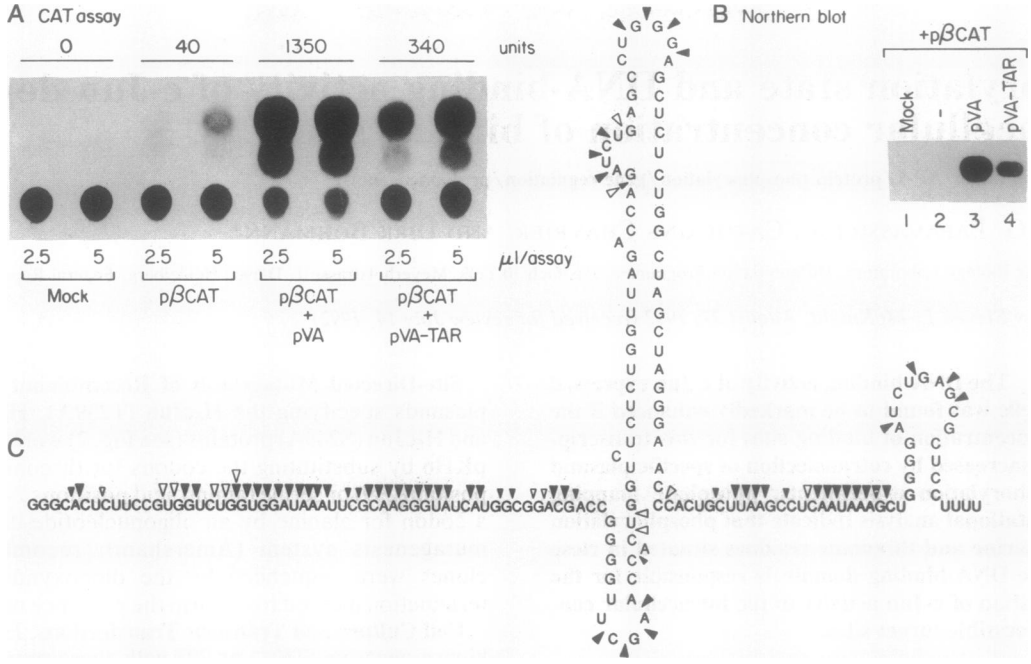

FIG. 5. Stimulation of gene expression *in vivo*. (*A*) CAT enzyme activity in lysates of human 293 cells (31) transfected with carrier DNA only (lanes 1, 2), 3 μg of pβCAT plasmid alone (lanes 3, 4), or with 5 μg of pVA (lanes 5, 6), or 10 μg of pVA-TAR (lanes 7, 8). Salmon sperm DNA (Pharmacia) was added as a carrier to a total of 13 μg per plate. Cells were harvested at 48-hr posttransfection, and CAT assays (32) were conducted with two different amounts of lysate. (*B*) RNA blot (33) of RNA from transfected cells probed with an RNA complementary to the 5′ end of VA RNA transcribed from p5′VA (34) cut at *Xba* I. Cells were transfected with salmon sperm DNA only (lane 1), 3 μg of pβCAT alone (lane 2), or with 5 μg of pVA (lane 3), or 10 μg of pVA-TAR (lane 4). (*C*) Secondary-structure model for VA-TAR RNA.

whether the short TAR transcripts accumulate in the infected cell to a sufficiently high concentration to block DAI activation. On the other hand, because DAI activation can be controlled at the local level (40–42), the possibility remains open that the TAR structure located at the 5′ end of full-length mRNAs protects HIV-1 protein synthesis against DAI activation, giving these viral mRNAs a selective advantage over cellular mRNA when dsRNA is present. The location of the TAR structure on each viral RNA would position it ideally for such a cis-acting translational role.

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