

# Tat-responsive region RNA of human immunodeficiency virus 1 can prevent activation of the double-stranded-RNA-activated protein kinase

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

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Communicated by Sidney Altman, August 22, 1990 (received for review July 3, 1990)

**ABSTRACT** Transcription from the human immunodeficiency virus type 1 promoter gives rise to short cytoplasmic transcripts of  $\approx 60$  nucleotides as well as to longer mRNAs. These RNAs contain the Tat-responsive region sequence, which is capable of assuming a stem-loop structure and has been implicated in the regulation of both transcription and translation. It has been reported that Tat-responsive region RNA inhibits translation *in vitro* through activation of an interferon-induced protein kinase, the double-stranded-RNA-activated inhibitor, which phosphorylates eukaryotic initiation factor 2. We show that the activation property is due to double-stranded RNA that often contaminates RNA synthesized *in vitro* using bacteriophage RNA polymerases. After purification, high concentrations of Tat-responsive region RNA inhibit the activation of double-stranded RNA-activated inhibitor, suggesting that it may serve to protect human immunodeficiency virus type 1 infection from a cellular defense mechanism.

The human immunodeficiency virus type 1 (HIV-1) produces three major size classes of translatable RNA during its life cycle (for reviews, see refs. 1–3). In the early stages of infection, the RNAs are multiply spliced and encode regulatory proteins, whereas late-stage RNAs are singly spliced or unspliced and encode structural proteins. One of the regulatory proteins, the trans-acting protein Tat, greatly stimulates the expression of HIV-1 products. Tat renders its effect through a sequence known as the Tat-responsive region (TAR) located downstream from the transcriptional initiation site in the 5' long terminal repeat (LTR) (1–3). Originally mapped between nucleotides –17 and +83, relative to the transcriptional start at +1, the essential region of the TAR element extends between nucleotides +19 and +42.

A transcript of the TAR sequence is present in the 5' untranslated region of all classes of HIV-1 mRNA. Transcription from the HIV-1 promoter also gives rise to a class of short promoter-proximal RNAs through a process that is regulated by Tat (4–7). These transcripts also contain the TAR sequence: they are  $\approx 60$  nucleotides long, nonpolyadenylated, and accumulate in the cytoplasmic fraction, but their significance is not known. The TAR RNA sequence is predicted to form a stable stem-loop structure comprising nucleotides +1 to +59 (8, 9). In addition, a second stem-loop structure, extending from +60 to +104, is predicted for longer HIV RNAs (9). Empirical evidence for the existence of this secondary structure comes from ribonuclease-sensitivity probing (9), and additional support can be drawn from mutagenic studies (6, 10–12) and comparisons with HIV-2 (10, 13). The stem-loop structure encompassing the TAR region plays an important role in Tat-mediated tran-

scriptional activation (6, 9–12, 14) and interacts directly with the Tat protein (15, 16).

In contrast to this positive transcriptional role, it has been reported that the TAR RNA structure exerts a negative effect on mRNA translation in cis and in trans. Secondary structure in the 5' untranslated region of mRNA lowers its translational efficiency in cis (17). Consequently, mRNA with a TAR leader sequence is translated poorly compared to that without a leader or with a leader in which the stem is interrupted (18). The stem structure of TAR RNA is also reported to inhibit the initiation of translation *in vitro* by activating the double-stranded-RNA (dsRNA)-activated inhibitor of protein synthesis (DAI) (19, 20). DAI (also known as dsl and p68) is an interferon-induced protein kinase that is normally found in an inactive latent form. When activated by dsRNA, it phosphorylates eukaryotic initiation factor 2 (eIF-2), which then sequesters an essential second initiation factor (eIF-2B or GEF) and blocks protein synthesis in trans (for reviews, see refs. 21–23).

Results presented here show that the DAI activation property associated with TAR RNA transcribed *in vitro* is not due to TAR RNA *per se*. After rigorous purification of the synthetic RNA, its ability to activate DAI was lost. On the contrary, at high concentrations, purified TAR RNA prevents the activation of DAI by dsRNA. By analogy with adenovirus virus-associated (VA) RNA, which also blocks DAI activation and renders adenovirus infection resistant to interferon (24, 25), TAR RNA may serve to neutralize a cellular defense mechanism during HIV-1 infection.

## MATERIALS AND METHODS

**Plasmid Construction.** The plasmid pEM-7 was constructed by E. Mathews and M. Laspia (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) to direct initiation by T7 RNA polymerase at nucleotide +1 of HIV-1. Nucleotides +21 to +82 of the HIV-1 LTR were excised from pU3R-III (26) and inserted between the *HincII* and *HindIII* sites of pUC118 with a *Bgl* II linker at the former site. The *Bgl* II–*Bam*HI fragment of this construct was replaced by the equivalent fragment from pET-7 containing the  $\phi 10$  promoter of bacteriophage T7 (27). Finally, complementary synthetic oligonucleotides corresponding to nucleotides +3 to +20 of the HIV-1 LTR were annealed and ligated between the *Stu* I site of the T7 promoter and the *Bgl* II site.

**TAR RNA Preparation.** pEM-7 was linearized at nucleotide +82 by digestion with *HindIII* and transcribed using T7 RNA

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Abbreviations: ds, double stranded; DAI, dsRNA-activated inhibitor; TAR, Tat-responsive region; HIV, human immunodeficiency virus; VA RNA, virus-associated RNA; LTR, long terminal repeat; eIF-2, eukaryotic initiation factor 2.

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polymerase purified as described (28). Transcription and RNA purification followed procedures devised in this laboratory (29). Briefly, TAR RNA labeled with [ $\alpha$ - $^{32}$ P]GTP was purified through three steps. It was first subjected to electrophoresis in a sequencing-type 7 M urea/8% polyacrylamide gel and the major radioactive band was eluted. The RNA was extracted with phenol/chloroform and precipitated with ethanol. This stage 1 TAR RNA was then resolved in a nondenaturing 8% polyacrylamide gel. After phenol/chloroform extraction and ethanol precipitation, the RNA recovered from this second gel (stage 2 TAR RNA) was loaded onto a cellulose CF-11 column (30) in 35% (vol/vol) ethanol/STE buffer (10 mM Tris-HCl, pH 8.0/100 mM NaCl/1 mM EDTA) and eluted with 15 or 22% ethanol/STE. RNA was precipitated from peak fractions and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4/1 mM EDTA), giving stage 3 TAR RNA. RNA concentrations were determined spectrophotometrically.

**Other RNAs.** Anti-TAR RNA was transcribed by SP6 RNA polymerase from plasmid pGEM23 (7) digested with *Rsa* I. SP6 polymerase reactions were as specified by the manufacturer (Promega) and the RNA was purified as described above. The resultant RNA was complementary to TAR RNA with 4 extra nucleotides (complementary to nucleotides -1 to -4 of the HIV-1 LTR) at the 3' end and 5 nucleotides from polylinker sequences at the 5' end. VA RNA<sub>1</sub> was synthesized *in vitro* and purified as described (29). Short dsRNAs were produced by symmetrical transcription of a polylinker (ref. 23; L. Manche and M.B.M., unpublished results). Reovirus dsRNA was kindly provided by A. Shatkin (Waksman Institute, Rutgers University, New Brunswick, NJ).

**Kinase Assays.** DAI was purified from the ribosome salt-wash fraction of interferon-treated 293 cells through the hydroxylapatite stage (23). Kinase assays were conducted in the presence of [ $\gamma$ - $^{32}$ P]ATP and the RNAs under test. Products were analyzed by electrophoresis in SDS/polyacrylamide gels and autoradiography (23).

**Ribonuclease Digestion.** RNA was incubated with RNase T1 (Calbiochem) for 30 min at 37°C in TE buffer at an enzyme/substrate ratio of 1:10 (wt/wt). After incubation for an additional 15 min with 0.5% SDS/proteinase K (Boehringer Mannheim; 0.2 mg/ml), RNA was recovered by phenol/chloroform extraction and ethanol precipitation in the presence of glycogen (20  $\mu$ g/ml) as carrier. The precipitate was dissolved in TE buffer and a fraction was analyzed in a 7 M urea/8% polyacrylamide gel to verify complete digestion of TAR RNA. RNA was digested with RNase III (phosphocellulose fraction; ref. 31) in a similar fashion except that 200 ng of RNA was incubated with 6 units of RNase III in a digestion buffer [20 mM Tris-HCl, pH 7.6/100 mM NH<sub>4</sub>Cl/10 mM magnesium acetate (32)].

## RESULTS

**DAI Activation by Partially Purified TAR RNA.** TAR RNA, corresponding to nucleotides +1 to +82 of the HIV-1 LTR, was synthesized *in vitro* using T7 RNA polymerase and was initially purified by electrophoresis through a denaturing polyacrylamide gel (stage 1 RNA). In agreement with previous results (19, 20), the partially purified RNA activated DAI, as evidenced by autophosphorylation of the enzyme itself (Fig. 1*a*) and phosphorylation of its substrate, eIF-2 (Fig. 2*a*, lane 3). Activation required much higher concentrations of TAR RNA than of dsRNA prepared from reovirus ( $\approx$ 40 times higher; Fig. 1*a*). Furthermore, unlike authentic dsRNA, which activates at low concentrations but fails to activate at high concentration ( $\approx$ 1  $\mu$ g/ml) (for review, see ref. 33), the partially purified TAR RNA continued to activate DAI at the highest concentrations tested (Fig. 1*a*). These observations could imply mechanistic differences between TAR RNA and

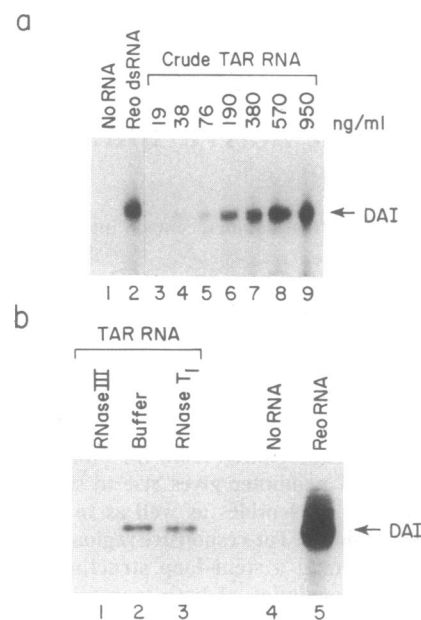


FIG. 1. DAI activation by crude (stage 1) TAR RNA. (a) DAI activation assays contained no RNA (lane 1), reovirus dsRNA (10 ng/ml; lane 2), or the indicated concentrations of stage 1 TAR RNA (lanes 3–9). (b) RNase sensitivity of the activation property of stage 1 TAR RNA. Reactions contained TAR RNA (200 ng/ml) treated with RNase III (lane 1) or RNase T1 (lane 3) or untreated (lane 2). Control assays contained no RNA (lane 4) or reovirus dsRNA (10 ng/ml; lane 5). The position of  $^{32}$ P-labeled DAI is marked.

longer dsRNAs in their interactions with DAI but could also reflect the presence of low levels of dsRNA contaminants in the stage 1 TAR RNA preparation.

To address the second possibility, we examined the sensitivity of the activation property toward the nucleases RNase T1 and RNase III. TAR RNA, being an imperfect stem-loop structure, is digested by the single-stranded RNA-specific enzyme RNase T1 (ref. 9 and see Fig. 4). RNase III, on the other hand, digests perfectly duplexed dsRNA >20 base pairs (bp) long (32) and is unable to act upon imperfectly paired duplexes unless they have a specific stem-bulge structure, as in some prokaryotic RNA processing sites (34). TAR RNA forms an imperfect 24-bp duplex (9) and is insensitive to RNase III digestion (Fig. 4, lanes 3 and 4). Fig. 1*b* shows that the capability of partially purified TAR RNA to activate DAI was unaffected by RNase T1 digestion but was ablated by incubation with RNase III. The reverse would be expected if TAR RNA itself were responsible for activation of DAI. Similar results were obtained with TAR-CAT fusion RNAs (19) transcribed by SP6 RNA polymerase (data not shown), suggesting that the activation of DAI by partially purified RNA is probably due to a dsRNA contaminant rather than TAR RNA itself.

**TAR RNA Loses the Ability to Activate DAI on Purification.** The partially purified TAR RNA, fractionated in a denaturing gel (stage 1 RNA), was further purified by electrophoresis through a nondenaturing gel (stage 2 RNA) and then by chromatography over a cellulose column under conditions designed to separate single-stranded RNA and dsRNA (stage 3 RNA). The kinase assays in Fig. 2*a* show that both DAI autophosphorylation and eIF-2 phosphorylation were drastically reduced when TAR RNA was purified to stage 2 or stage 3. Little or no activity was observed when the fully purified (stage 3) TAR RNA was tested over a wide range of concentrations (Fig. 2*b*). In case the RNA remained partially denatured after the purification, we heated samples of stage 3 RNA to 95°C and allowed them to cool slowly in the presence of potassium chloride, magnesium chloride, or both

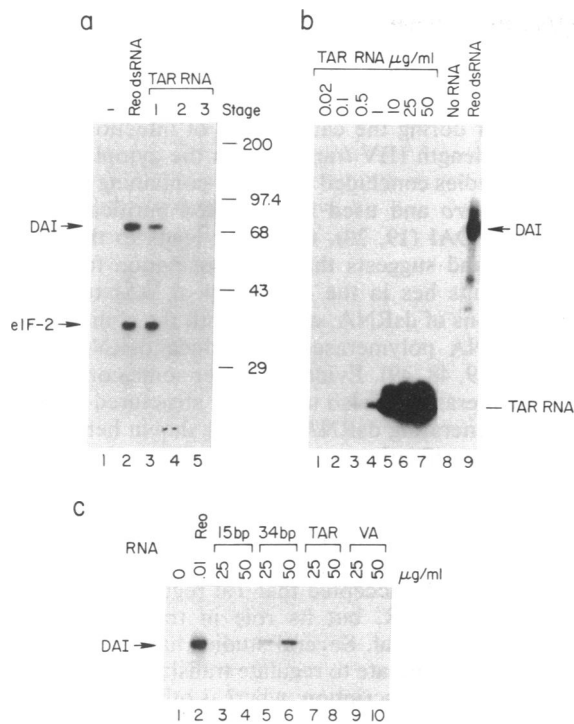


FIG. 2. DAI activation capacity of purified TAR RNA. (a) The kinase activation reactions contained eIF-2 and TAR RNA (200 ng/ml) at different stages of purification (lanes 3–5). The positions of DAI, the  $\alpha$  subunit of eIF-2, and molecular mass standards are indicated. Lane 1 contained no RNA and lane 2 contained reovirus dsRNA. (b) Kinase assays contained increasing concentrations of purified TAR RNA (stage 3), as indicated. (c) Comparison of activation capabilities of stage 3 TAR RNA (lanes 7 and 8) and adenovirus VA RNA<sub>1</sub> (lanes 9 and 10) with 15-bp dsRNA (lanes 3 and 4) or 34-bp dsRNA (lanes 5 and 6). Control assays contained no RNA (lane 1) or reovirus dsRNA (10 ng/ml; lane 2).

to allow the native conformation to reform. The treated TAR RNA samples also failed to activate DAI (data not shown). These data indicate that the ability to activate DAI is not an intrinsic property of TAR RNA.

Earlier studies suggested that the minimum length of dsRNA capable of activating DAI is 50 bp (35), but recent work indicates that shorter duplexes can activate, albeit to a lesser degree, provided they are at least 30 bp long (L. Manche and M.B.M., unpublished results). The experiment shown in Fig. 2c compares the ability of various short RNA duplexes to elicit DAI autophosphorylation. A perfect 34-bp duplex exhibited low but significant activity whereas the activity of a similar 15-bp duplex was barely detectable. In keeping with these findings, TAR RNA and adenovirus VA RNA<sub>1</sub>, which have no more than 11 bp of continuous duplex in their stem regions, were also barely active in this assay. Thus, there is no evidence that a special ability to activate DAI is conferred upon TAR RNA by its structure or sequence.

**TAR RNA Can Block DAI Activation.** Some RNA species that fail to activate DAI are nevertheless able to interact with the enzyme in such a way as to prevent its activation. The ability to block DAI autophosphorylation and activation is exhibited by short dsRNAs and by some small highly structured RNAs such as VA RNA<sub>1</sub> (24, 25, 33, 35) and possibly the Epstein-Barr encoded small RNAs (EBERs) of Epstein-Barr virus (36). To explore the possibility that TAR RNA functions in this way, various concentrations of reovirus dsRNA and TAR RNA were incubated with DAI. As shown in Fig. 3, TAR RNA blocked activation of DAI by dsRNA in a concentration-dependent fashion. At higher concentrations

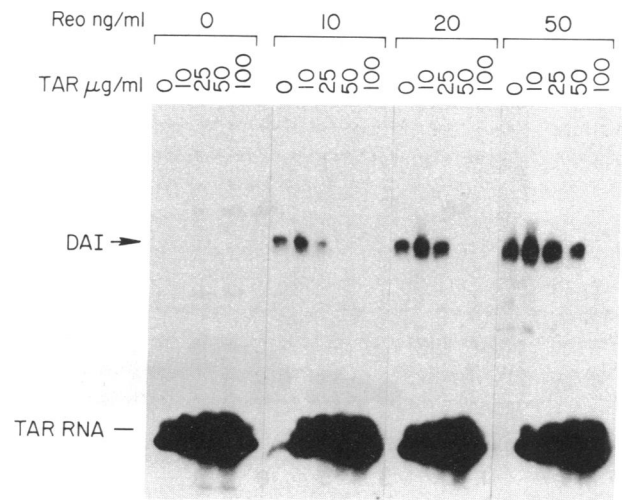


FIG. 3. TAR RNA blocks activation of DAI by dsRNA. DAI assays were performed in the presence of reovirus dsRNA and stage 3 TAR RNA at the concentrations indicated.

of dsRNA, more TAR RNA was required to block activation. To ensure that the inhibition is due to TAR RNA, we examined the nuclease sensitivity of the inhibitor. Fig. 4 shows that the RNase sensitivity of the inhibitory component matches that of TAR RNA: both were eliminated by RNase T1, but neither was affected by RNase III. Thus, the inhibition is an intrinsic property of TAR RNA.

At very high concentrations (>50 µg/ml), RNA can prevent the activation of DAI without any apparent specificity (ref. 24 and unpublished results). To assess the specificity of the inhibition by TAR RNA, the complementary sequence (anti-TAR RNA, nucleotides +82 to -4) was synthesized *in vitro* and purified through the three-step purification described above. Anti-TAR RNA did not inhibit DAI activation except at 100 µg/ml (compare Fig. 5, lanes 3–5, with lanes 6–8). We conclude that TAR RNA is able to serve as a specific inhibitor of DAI activation.

## DISCUSSION

The protein kinase DAI is one component of the cellular anti-viral defenses (37, 38). Its synthesis is elevated by

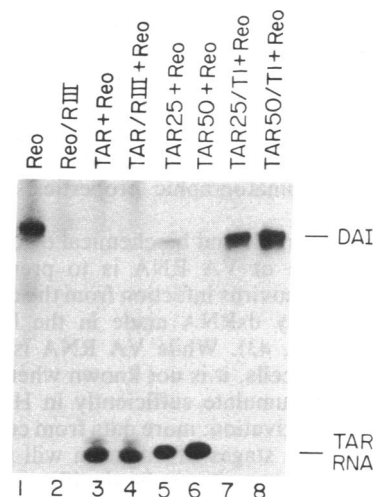


FIG. 4. RNase sensitivity of TAR RNA and DAI activation. DAI assays were performed in the presence of reovirus dsRNA at 10 ng/ml alone (lanes 1 and 2) or with stage 3 TAR RNA at 25 µg/ml (lanes 5 and 7) or 50 µg/ml (lanes 3, 4, 6, and 8). The RNAs were treated with RNase III or RNase T1 as indicated.

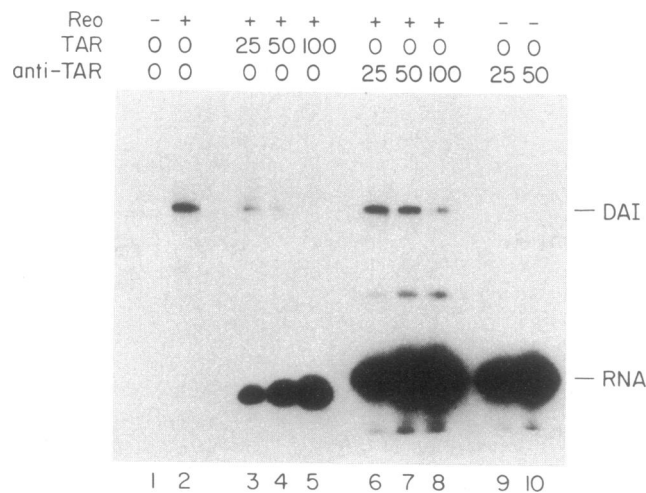


FIG. 5. Effect of anti-TAR RNA on DAI activation. DAI assays were performed with reovirus dsRNA, stage 3 TAR RNA, and stage 3 anti-TAR RNA, at the concentrations indicated. -, Reovirus dsRNA absent; +, reovirus dsRNA present.

interferon, and its activity is unmasked by dsRNA, which is a frequent concomitant of viral infection. Activation of the enzyme blocks protein synthesis by phosphorylation of an initiation factor, eIF-2, which is one of its few known substrates. DAI is activated by very low concentrations of dsRNA (33) and uninfected cells may possess detoxification mechanisms to dispose of dsRNA that accumulates as a result of normal metabolism (39, 40). Presumably, these safety mechanisms are saturated or otherwise incapacitated during viral infection, allowing dsRNA to accumulate and trigger the activation of DAI and other dsRNA-dependent enzymes. For their part, several viruses have acquired the means to evade this anti-viral defense (41). These countermeasures involve viral proteins, as in influenza, polio-, reo-, and vaccinia viruses, and viral RNAs, as in adenovirus and perhaps Epstein-Barr virus. The work presented here raises the possibility that the HIV-1 TAR RNA may also play such a role.

Present at the 5' end of all HIV mRNAs and in a free cytoplasmic form, the TAR sequence adopts a stem-loop structure that can interact with DAI. Being <30 bp long, the TAR RNA duplex fails to activate DAI as longer perfect duplexes do, but at high concentration it prevents DAI activation. In this respect it resembles adenovirus VA RNA<sub>1</sub>, a 160-nucleotide transcript synthesized by RNA polymerase III (24, 25, 33). Both of these species of small stable cytoplasmic viral RNA are highly structured even though they behave as predominantly single-stranded molecules with regard to their chromatographic properties and nuclease sensitivity (9, 42).

A large body of genetic and biochemical evidence demonstrates that the role of VA RNA is to preserve protein synthesis during adenovirus infection from the consequences of DAI activation by dsRNA made in the late phase of infection (21, 24, 25, 43). While VA RNA is abundant in adenovirus-infected cells, it is not known whether the short TAR transcripts accumulate sufficiently in HIV-1-infected cells to block DAI activation: more data from cells of various types and in various stages of infection will be needed to settle this issue. However, it may be significant that the TAR sequence is also carried by HIV mRNAs, in an ideal position to spare them from the inhibitory effect of DAI activation on polypeptide chain initiation. DAI is bound to ribosomes, and the idea that its activity can be controlled at a local level has precedents *in vivo* and *in vitro* (44, 45). Thus, it has been proposed that VA RNA ensures the preferential translation of

mRNAs with which it is specifically associated (45-47). Similarly, by keeping DAI inactive, the TAR sequence at the 5' end of HIV mRNAs may allow them to be translated preferentially. This may be important in the presence of interferon or during the early stages of infection when the level of full-length HIV transcripts in the cytoplasm is low.

Earlier studies concluded that TAR-containing RNA, transcribed *in vitro* and used after limited purification, is an activator of DAI (19, 20). Our work leads to the opposite conclusion and suggests that the explanation for the contrasting results lies in the sensitivity of DAI to very low concentrations of dsRNA, coupled with the ability of bacteriophage RNA polymerases to produce dsRNA in small quantities (29, 48, 49). Evidently under some conditions, T7 RNA polymerase can also use highly structured RNAs as a template, generating dsRNA (50). As shown here and elsewhere (29), purification over a denaturing gel may not adequately remove dsRNA contamination. When this was accomplished by further steps of purification, the activation capacity of the TAR RNA was lost and its ability to block activation was revealed.

It is now widely accepted that Tat regulates transcription from the HIV LTR, but its role in translational control remains controversial. Several studies have concluded that Tat and TAR cooperate to regulate translation, either as well as or instead of transcription, whereas other studies failed to discern an effect at the translational level (1, 3). The findings described here may provide a basis for reconciling these discrepancies. Basal levels of DAI vary greatly among cell types and possibly as a result of growth conditions (38). Accordingly, the requirement for VA RNA<sub>1</sub> during adenovirus infection is reduced or eliminated when the DAI concentration is low (45, 51). The requirement is also influenced by a number of other factors, including eIF-2 mutations (52, 53), trans-acting proteins (54), and mechanisms that are presently unknown (55). On the other hand, in transfection experiments, factors that lead to dsRNA accumulation increase the requirement for VA RNA (47, 56). If TAR RNA acts like VA RNA to block the activation of DAI, the expression of HIV transcripts at a high level (for example, because of the presence of Tat) might increase protein synthesis in some cell lines or experimental conditions but not in others. This may account, at least in part, for the variability reported in the literature.

Finally, in addition to immunological abnormalities, AIDS is accompanied by a suppression of the interferon-mediated defense system. For example, interferon- $\alpha$  and - $\gamma$  production declines and exogenously administered interferon- $\alpha$  fails to augment (2'-5')oligoadenylate synthetase levels (57). Moreover, a decrease in DAI levels has been observed in HIV-infected cells (58). Since HIV-1 replication is sensitive to interferon, the inhibition of DAI activation by TAR RNA may be seen as a further aspect of the disarming of host defenses by the virus.

We thank T. Pe'ery, K. Mellits, and M. Laspia for advice and discussions, N. Sonenberg for suggestions and SP6 TAR/CAT plasmids, and L. Manche for DAI. This work was supported by National Institutes of Health Grants CA13106 to M.B.M., AI25308 and 27270 to A.P.R., and GM28294 to H.D.R.

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