JOURNAL OF VIROLOGY, Jan. 1994, p. 25–33
0022-538X/94/$04.00+0

Vol. 68, No. 1

Inhibition of Human Immunodeficiency Virus Type 1 Tat-Dependent Activation of Translation in *Xenopus* Oocytes by the Benzodiazepine Ro24-7429 Requires *trans*-Activation Response Element Loop Sequences

MARTIN BRADDOCK, PAULA CANNON, MARTINA MUCKENTHALER, ALAN J. KINGSMAN, AND SUSAN M. KINGSMAN*

Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

Received 12 May 1993/Accepted 24 September 1993

Two benzodiazepine compounds, [7-chloro-5-(2-pyrryl)-3H-1,4 benzodiazapin-2-(H)-one] (Ro5-3335) and [7-chloro-5-(1H-pyrrol-2-yl)-3H-benzo[e] [1, 4] diazepin-2-yl]-methylamine (Ro24-7429), inhibit human immunodeficiency virus type 1 (HIV-1) replication via a specific effect on the function of the transactivator protein, Tat. To gain further insight into the mechanism of action of these compounds, we have tested their effects in an alternative assay for Tat activation in *Xenopus* oocytes. In this system, translation of *trans*-activation response element (TAR)-containing RNA is activated by Tat. Both compounds specifically blocked activation of translation in a dose-dependent fashion, with Ro24-7429 showing the greater potency. In the *Xenopus* oocyte system, as in mammalian cells, mutation of the TAR loop sequences abolishes Tat action. However, it is possible to obtain TAR-specific, Tat-dependent activation of a target RNA with a mutation in the loop provided that this target is in large excess. This result has been interpreted as indicating that a negative factor has been titrated (M. Braddock, R. Powell, A. D. Blanchard, A. J. Kingsman, and S. M. Kingsman, FASEB J. 7:214–222, 1993). Interestingly Ro24-7429 was unable to inhibit the TAR-specific but loop sequence-independent mode of translational activation. This finding suggests that a specific loop-binding cellular factor may mediate the effects of this inhibitor of Tat action. Consistent with this notion, we could not detect any effect of Ro24-7429 on the efficiency of specific Tat binding to TAR in vitro.

Efficient replication of human immunodeficiency virus (HIV) is critically dependent on the virally encoded transactivator protein, Tat. Tat stimulates transcription by interacting with a specific sequence, the *trans*-activation response element (TAR). TAR is a stable RNA structure located at the 5′ end of all HIV mRNAs (12, 37) and comprises a partially base-paired stem, a tripyrimidine bulge, and a hexanucleotide loop (17, 32). In vitro binding studies have shown that Tat binds to TAR at the bulge (16, 48, 47) via an arginine-rich sequence in Tat (10, 11, 13, 36). The loop sequence in TAR is not important for Tat binding in vitro; however, many in vivo studies have indicated that mutations in the TAR loop sequence abolish Tat activation (e.g., reference 18). This is widely interpreted as suggesting that TAR-binding cellular factors are required in addition to Tat to achieve activation. Several cellular proteins that bind specifically to the loop in vitro have now been described; these are p68 (30), p185 (50), and TRP1 (probably the same as p185) (43). Several other TAR-binding proteins have been identified. These are a family of 70- to 110-kDa proteins (TRP2 family) that bind to the bulge region (43), a 37-kDa protein (TRBP1) that requires the upper stem but not the loop or the bulge for binding (19), a 38-kDa protein (bulge-binding protein) that requires the bulge and residue G-26 but not A-27 for maximal binding (31a), the DAI kinase, which binds to the lower stem (21), and stem-binding protein, which also binds to the lower stem, the bulge, and A-27 of the upper stem (39). There is also evidence for additional factors, but these are as yet poorly characterized (31). A number of Tat-binding proteins have also been identified (14, 33, 34). All of these observations suggest that Tat activation in vivo may require not only Tat binding to TAR but also the interactions of cellular factors with TAR and with Tat.

Although the predominant mode of action of Tat is to stimulate transcription, we have observed that Tat can specifically activate the translation of TAR-containing RNAs after they have been injected into *Xenopus* oocytes. This activation requires nuclear factors and shows the same specificity with respect to loop, bulge, and upper stem sequences as does transcriptional activation in mammalian systems (5–9, 13, 18, 27). Although the loop sequence is important, it is possible to establish loop sequence-independent activation in the oocyte system. A TAR RNA target with a mutation in the loop can be activated by Tat provided that the target is present at very high intracellular concentrations. This activation is abolished by second-site mutations in the bulge, indicating that it is still dependent on the specific Tat-TAR binding interaction (7). We have suggested that under these conditions, a negative factor has been titrated, and we proposed that the loop sequences are critical only in the presence of this putative negative factor. This contrasts with other suggestions that the loop sequence is essential to recruit a coactivator protein (28, 29). Irrespective of the explanation for the role of the TAR loop, our data show that it is possible to assay a specific facet of Tat action in an in vivo system in which the loop sequences and, by inference, loop sequence-binding cellular factors do not play a role.

The pivotal importance of Tat activation to ensure efficient productive viral replication has stimulated the search for specific inhibitors of this process. Recently a class of benzodiazepines that inhibit HIV replication and which also specifi-

* Corresponding author. Mailing address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom. Phone: 865 275248. Fax: 865 275259.

cally block Tat activation of transcription from HIV promoter-reporter plasmids has been identified (23, 24, 41, 49). To date, there are few insights into the mechanism whereby these compounds inhibit Tat activation. Clearly, they could function to directly block Tat binding, they could antagonize positive cellular factors which might bind to either TAR or Tat, or they could stimulate inhibitory factors. Recently it has been shown that prolonged passage of virus in the presence of these compounds does not select for drug-resistant viruses, suggesting that the target for these compounds could be cellular factors (23). In this report, we provide more information about the antiviral activity of Ro24-7429. In addition, we use the *Xenopus* oocyte assay system to probe for possible interactions between these drugs and cellular factors. Our data show that the compounds block Tat-specific activation of translation but that their effect is dependent on the integrity of the TAR loop sequence. These data suggest that these benzodiazepines interact with loop-binding cellular factors.

MATERIALS AND METHODS

Plasmids and in vitro transcription. Plasmid constructions have been previously described (7). Derivatives of pSP65 that generated wild-type (pPE38) and loop mutant (pPE43) chloramphenicol acetyltransferase (CAT) reporter RNAs, referred to as 38 RNA and 43 RNA, respectively, were constructed. Short competitor RNAs (CTARs) were produced by runoff at the unique *Nhe*I site as described previously (7). Plasmids, transcripts, and TAR sequences are shown in Fig. 1. In vitro transcription was performed as described previously (8), and all procedures were carried out as recommended by the supplier (Promega, Madison, Wis.). All RNAs were capped as previously described (8). A nonspecific competitor RNA was produced as described previously (7).

RNA and protein analysis. RNA was extracted from oocytes and analyzed by quantitative primer extension as previously described (7, 9), using a primer located in the CAT reporter RNA (5). Primers were end labelled with [γ-^{32}P]ATP, and extension products were visualized on 8% denaturing polyacrylamide gels (5). CAT assays were done as described previously (7), using typically 50 μg of protein for a 1-h CAT assay. CAT assays and primer extension analyses were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis without file conversion (25). In all experiments, oocyte extracts were prepared for RNA and protein analysis 16 to 18 h after injection.

***Xenopus* oocyte microinjection.** *Xenopus* oocytes were isolated and maintained in Barth X medium as described previously (7). Tat activation conditions used 3 ng of reporter RNA and 20 pg of Tat injected per oocyte. Typically 30 oocytes were used to generate each datum point, and each observation was confirmed in at least three independent experiments using different batches of oocytes. In competition experiments, reporter RNA was premixed with Tat and competitor RNA as previously described (7).

Compounds. Ro5-3335 and Ro24-7429 were synthesized as described previously (3). 9-Chloro TIBO was purchased from Pharmatech Inc., N.J. All compounds were dissolved in 100% dimethyl sulfoxide (DMSO) to 7.4 mM. For antiviral studies, the compounds were added to tissue culture medium to give the final stated concentrations. For *Xenopus* oocyte studies, the compounds were added to Barth X buffer, and they were coinjected into oocytes and maintained in the medium at the stated concentrations. In dose titrations, the final concentration of DMSO was constant across the dilution range. In control experiments in both mammalian and *Xenopus* studies, DMSO was present in the medium at the same concentration as used in the drug experiments; no effects of DMSO at these concentrations were observed.

Antiviral assays. HIV-1 virus stocks were prepared by DEAE-dextran transfection of H9 cells with the proviral clone WI3 (26) as described previously (1). Supernatants were harvested at 9 days posttransfection, filtered, and assayed for infectivity by a 50% tissue culture infectious dose assay on C8166 cells (1). For acute infections, 10^7 H9 cells were incubated with 10^6 50% tissue culture infectious doses of HIV-1 virus in 1 ml of medium for 1 h at 37°C and then washed and resuspended in fresh medium at 10^5 cells per ml. TIBO was added to the cells at a final concentration of 1 μM. Ro24-7429 was added to the cells at a final concentration of 0.1 or 0.2 μM, as stated. An equivalent volume of DMSO was added to the control population. Samples of the culture supernatant were taken when the cells were passaged and then assayed for reverse transcriptase activity as described previously (1). H9 cells chronically infected with HIV-1 were established 20 days after the initial infection with WI3, when reverse transcriptase activity had reached a steady level. Then 10^6 cells were plated in 5 ml of fresh medium, and Ro24-7429 was added to give a final concentration of 0.1 or 0.2 μM; a control population had an equivalent amount of DMSO added. Three days later, the supernatants were assayed for reverse transcriptase activity and the number of viable cells in each sample was calculated by trypan blue exclusion.

Tat binding. Tat was produced as an N-terminal hexahistidine-tagged protein as described previously (20). Tat was bound to TAR for 15 min at 23°C in Tat-binding buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 50 mM NaCl, 0.5 U of RNasin per ml, 0.04 μg of bovine serum albumin per ml, 0.05% glycerol) in a final volume of 20 μl. TAR RNAs were prepared by in vitro transcriptions (7). Short +17-to-+43 TAR RNAs were constructed by subcloning an oligonucleotide containing the wild-type HIV-1 TAR sequence 5′-ATTCCAGATCTGAG CCTGGGAGCTCTCTGGA-3′ or the corresponding mutant sequences into the *Eco*RI and *Hin*dIII sites of pGEM3Zf+ (Promega). Short TAR RNA sequences were flanked by the polylinker sequence 5′-CTTATGAGTTCGAA-TAR-G-3′. To confirm the specificity of Tat binding to TAR RNA, a triple-base substitution in the bulge, UCU to CUC, and a triple-base substitution in the loop, CUGGGA to AGGGUA, were also used in binding assays. The identity of the plasmids was confirmed by dideoxy sequencing (40). RNA from wild-type and mutant constructs was prepared from *Hin*dIII-linearized templates by using T7 polymerase (Bethesda Research Laboratories). All labelled and nonlabelled RNAs were synthesized as specified in the Promega manual. The labelled RNAs were purified from a denaturing 6% polyacrylamide gel, eluted overnight into 0.5 M sodium acetate (pH 7.0)–1 mM EDTA–0.5% sodium dodecyl sulfate, and precipitated with 0.3 M NaCl and 2.5 volumes of ethanol.

Ro24-7429 was added to the binding reactions at concentrations stated in the text. The compounds were preincubated with Tat in Tat-binding buffer for 8 min prior to addition of the TAR RNA probes. Addition of Ro24-7429 after the Tat-TAR binding reaction was established or preincubation of the compound with the TAR RNA probe did not alter the outcome (data not shown).

RESULTS

Ro24-7429 inhibits replication of HIV-1 in acute and chronic infections of H9 cells. The potency of Ro24-7429 was tested by examining its effect on the levels of reverse tran-

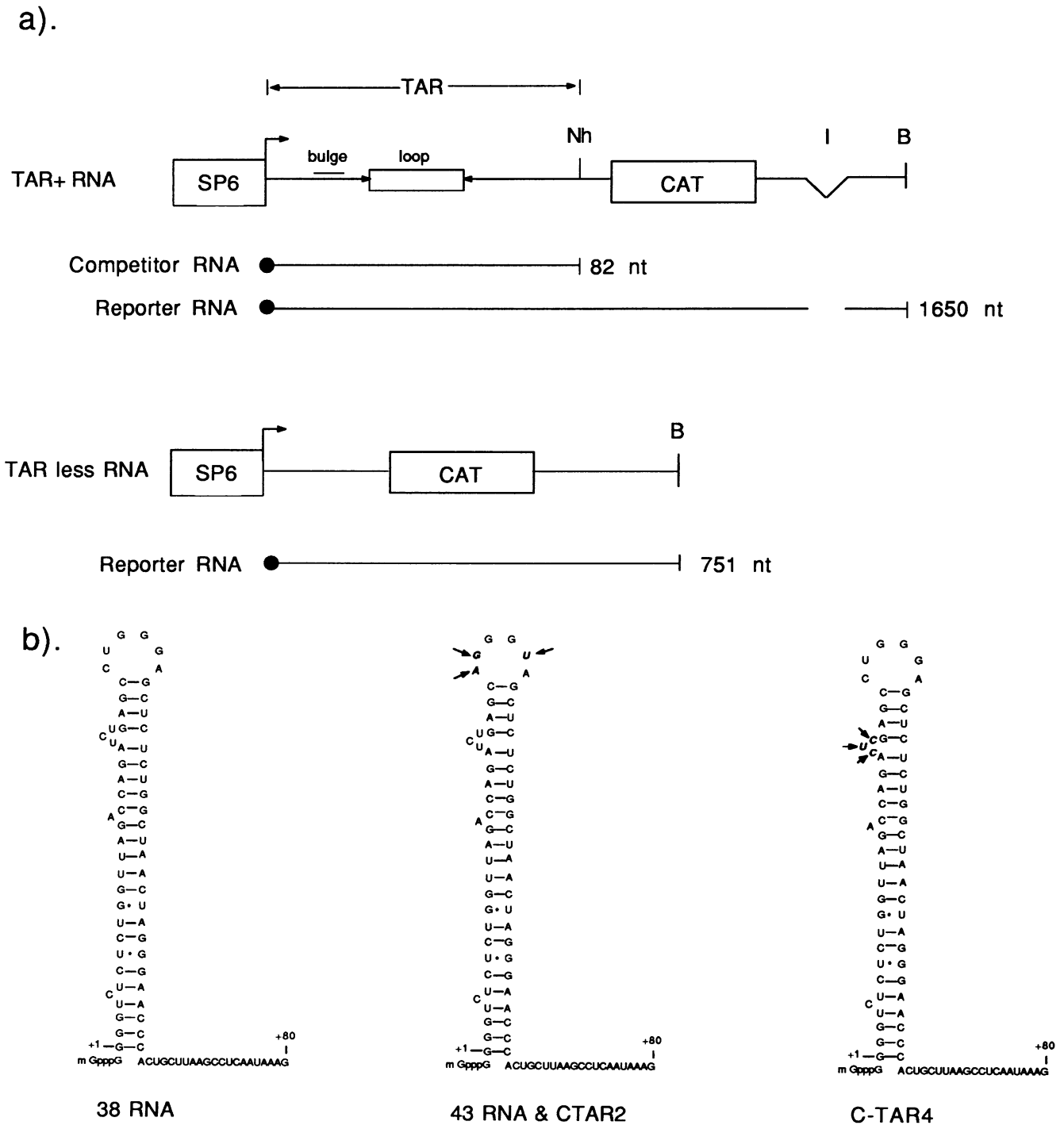

FIG. 1. Structures of synthetic reporter and competitor RNAs. (a) General structures of the SP6 templates used to produce TAR-plus or TAR-less reporter RNAs. Reporter RNAs were run off at the unique *Bam*HI site. SP6, SP6 promoter; CAT, bacterial CAT coding region; I, simian virus 40 small-t intron; B, *Bam*HI; Nh, *Nhe*I; nt, nucleotides. TAR domains are indicated. Inverted arrows, stem; line, bulge; open box, loop. (b) Structures of the TAR sequence in reporter and competitor RNAs. Competitor RNAs CTAR2 and CTAR4 were run off at the unique *Nhe*I site in plasmids pPE43 and pPE705 (7). For reference, the sequence of the TAR region in pPE38 is also shown. A nonspecific competitor derived from plasmid pSP72 was prepared as described previously (7).

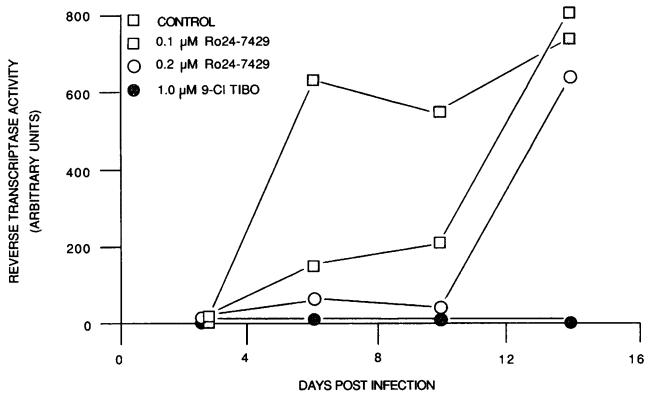

FIG. 2. Effect of Ro24-7429 on HIV-1 replication in acutely infected H9 cells. The graph show the levels of reverse transcriptase activity measured at various times after infection of H9 cells with HIV-1 and continuously treated with either Ro24-7429 or 9-chloro TIBO.

scriptase produced by acutely infected or chronically infected H9 cells. In the acute assays, the benzodiazepine derivative 9-chloro TIBO was used as a control for virus inhibition. This benzodiazepine compound has potent antiviral activity, but it functions as an inhibitor of reverse transcriptase rather than as an anti-Tat compound (35). In keeping with one other report (49), we were unable to rigorously test the antiviral activity of Ro5-3335 because of the extreme toxicity of this compound for all cells that we tested such that a dose that was antiviral but not cytotoxic could not be achieved. In the case of Ro24-7429, preliminary titrations showed no toxicity up to a concentration of 0.5 μM, and therefore antiviral activity was tested at concentrations below this. As shown in Fig. 2, in acute infections, Ro24-7429 was inhibitory at 0.1 and 0.2 μM when assayed at early times after initial infection. However, despite maintenance of the drug at this concentration by replenishment with fresh medium, by 10 days after infection significant viral replication was evident, and by 14 days it had reached control levels. Breakthrough replication was slightly delayed at the higher drug concentrations, indicating a dose-dependent effect. In contrast, no viral replication was detected during the course of the experiment using 9-chloro TIBO. In chronic infections, Ro24-7429 reduced reverse transcriptase levels to 48% of untreated levels at 0.1 μM and to 35% of untreated levels at 0.2 μM. These data indicate that Ro24-7429 has potent antiviral activity in the initial stages of infection but that virus escape occurs. Because of the short duration of the experiment, this result is highly unlikely to reflect the selection of resistant viruses but suggests that it is difficult to achieve complete inhibition of Tat activation with this compound.

Ro24-7429 and Ro5-3335 specifically inhibit Tat activation of translation. The ability of Ro5-3335 and Ro24-7429 to inhibit Tat activation of a long terminal repeat reporter plasmid has been documented (23, 24, 49). We have also shown that Ro24-7429 reduces the levels of Tat-activated transcription to a greater extent than it affects basal HIV transcription or general transcription (10a). However, in keeping with other observations with Ro5-3335 (49), we noted that with Ro24-7429, the specificity index was low. In our hands, in H9 and HeLa cells, it was about threefold. It was also not possible to completely abolish Tat activation at doses that were not cytotoxic. To explore the anti-Tat properties of these compounds further, we therefore decided to test the ability of these compounds to inhibit Tat-mediated activation of translation in the *Xenopus* oocyte system.

To demonstrate Tat activation of translation, it is necessary to coinject Tat and the TAR RNA target into the nucleus (6). RNA synthesized in vitro which contains TAR fused to a CAT reporter coding sequence (38 RNA; Fig. 1) was injected into the nucleus of *Xenopus* oocytes with Tat and with Tat plus 0.37 or 37 μM Ro5-3335 and Ro24-7429. As shown in Fig. 3a, in the absence of any drug, the typical high level of activation by Tat was observed (lanes 1 and 2). However, activation was reduced about fivefold (lane 3) in the presence of 0.37 μM Ro5-3335 and was abolished at 37 μM (lane 5). Ro24-7429 abolished activation at both concentrations (lanes 4 and 6). In a control experiment, the compounds had no effect on the translation of a TAR-minus CAT reporter (151 RNA) in a conventional translation assay (lanes 7 to 11).

To characterize these anti-Tat effects further, we carried out a dose-response analysis of the effect of Ro24-7429. Translational activation of TAR-RNA by Tat was progressively inhibited by an increasing concentration of Ro24-7429 until complete inhibition was observed at a dose of 3.7 μM (Fig. 3b, lanes 1 to 6). From these and similar data, the concentration required to inhibit activation by 50% was about 0.03 μM (Fig. 3d). This was somewhat lower than the value in antiviral assays, which has been reported as 0.1 to 1 μM (19) for Ro5-3335 and is about 0.2 to 0.3 μM for Ro24-7429 when measured at day 6 in an acute infectivity assay as described above (Fig. 2a and unpublished data). It is likely that the greater potency of these compounds in *Xenopus* oocytes simply reflects the ability to deliver a higher effective dose to the nucleus via direct injection. Ro24-7429 did not inhibit the translation of the control RNA (151 RNA) at any dose point (Fig. 3a, lanes 11 to 17). Primer extension analysis of extracted RNA excluded the possibility of the drug influencing RNA stability, as the lack of expression did not reflect a lack of RNA (Fig. 3c). In contrast to Ro24-7429, the benzodiazepine derivative 9-chloro TIBO (35) did not inhibit translational activation by Tat in this system (compare lanes 9 and 10 in Fig. 3b; see also Fig. 5b, lane 4). These data suggest, therefore, that the inhibition of Tat activation of translation observed with Ro24-7429 is specific to the Tat-TAR interaction and to this particular class of benzodiazepine compounds.

Ro24-7429 fails to inhibit Tat-mediated translational activation of a loop-defective TAR RNA. To gain further insight into the mechanism of action of Ro24-7429, we decided to test its effect in two genetic assays that we have recently developed in *Xenopus* oocytes for TAR-specific activation in the absence of potential specific loop-binding factors. These assays and their interpretation have been presented and discussed in detail by Braddock et al. (7) and are briefly outlined below and in Fig. 4.

If the loop sequence in TAR is mutated, then translational activation by Tat is abolished (6) [Fig. 4A(i)]. However, we have shown that the requirement for the TAR loop sequence in this type of assay is conditional and not absolute (7). This was most simply demonstrated by showing that a TAR loop mutant reporter RNA (43 RNA) could be activated by Tat in the presence of a large excess of a short TAR loop mutant competitor RNA (CTAR2; Fig. 1b). A TAR RNA with mutations in the loop and the bulge did not allow activation of the loop mutant reporter RNA. A TAR reporter with a mutation in the loop and the bulge could not be activated by Tat in the presence of excess CTAR2, indicating that the activation of a loop-mutated TAR element was still dependent upon Tat interacting with the bulge sequence. We have devised a simple model that explains these observations. We propose

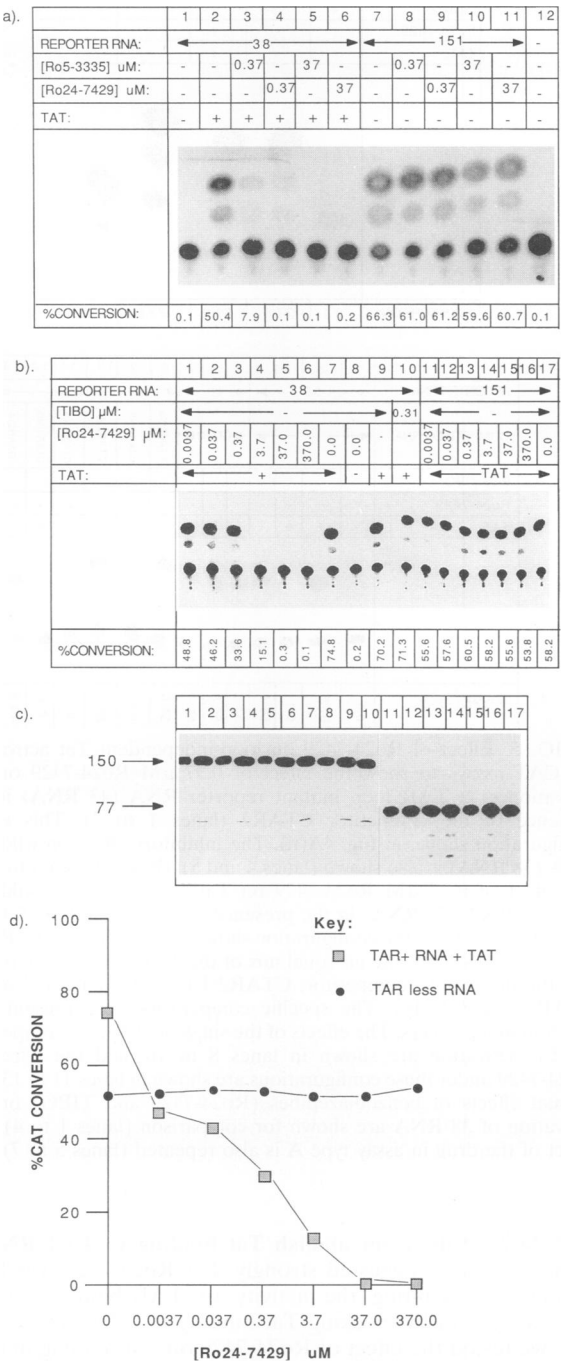

FIG. 3. Effects of benzodiazepines on Tat-mediated activation of translation in *Xenopus* oocytes. (a) CAT assays to show the effects of Ro5-3335 and Ro24-7429 on translation of TAR-plus RNA (38 RNA; lanes 1 to 8) and TAR-less RNA (151 RNA; lanes 7 to 12). (b) CAT assays to show a dose-dependent inhibition of Tat activation by Ro24-7429 (lanes 1 to 8) and to show that TIBO has no inhibitory effects (lane 10). Lanes 11 to 17 show the lack of inhibition of control RNA. Activation by Tat in the absence of drugs is shown in lane 9. (c) Analysis of RNA samples corresponding to the CAT assay samples in panel b. Sizes of primer extension products are indicated in nucleotides. (d) Graphical representation of the data in panel b.

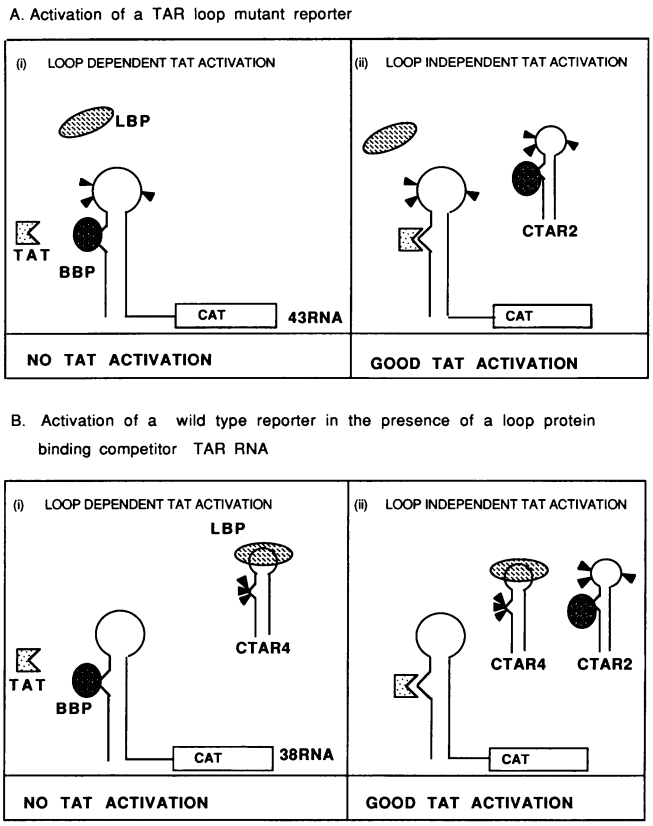

FIG. 4. Assay systems for TAR-specific but loop-independent activation by Tat. Protein interactions with TAR RNA under different conditions are indicated. These interactions have been deduced from in vivo genetic analyses (7). The arrowheads indicate sites of mutation in TAR; these are shown in the sequences in Fig. 1b. The proteins are Tat, a loop-binding protein (LBP), and a bulge-binding protein (BBP). Assay A shows a loop mutant reporter, 43 RNA, activated by Tat. In panel (i), the normal interactions with this reporter are shown; these result in no activation by Tat. In panel (ii), the interactions in the presence of excess competitor RNA, CTAR2, are shown. This configuration allows Tat activation. Assay B shows a wild-type reporter, 38 RNA, activated by Tat in the presence of competitor TAR RNA that removes the loop-binding protein. In panel (i), the normal interactions are shown whereby a competitor, CTAR4, blocks Tat activation by virtue of removing loop-binding proteins. In panel (ii), the interactions are shown in the presence of the competitor, CTAR4, and the counter competitor, CTAR2. This configuration allows Tat activation.

that there is a negative factor that binds specifically to the Tat binding site at the bulge and that this was being removed by binding to the loop mutant competitor TAR RNA [Fig. 4A(ii)]. The role of the loop therefore becomes one of protecting TAR RNA from this negative factor; once the factor is removed, the loop sequence is redundant.

A second type of assay also leads to the conclusion that the activation of wild-type TAR RNA can occur in the absence of the loop sequences. Tat activation of a wild-type TAR RNA can be abolished by competition with excess of a TAR RNA with a mutation in the bulge region (CTAR4). Given that a TAR RNA with this structure does not bind Tat (13, 16, 17), this finding suggests that an essential cellular factor is being removed, and this factor is likely to be a loop-binding protein(s) [Fig. 4B(i)]. In contrast, a TAR RNA with a mutation in the loop (CTAR2) has no function as a competitor, implying

that it binds neither to Tat nor to any positive cellular factors. If, however, the two competitors (CTAR2 plus CTAR4) are used together, then the ability of the bulge mutant competitor (CTAR4) to reduce Tat activation is abolished and Tat-dependent activation is observed. The loop mutant competitor (CTAR2) is therefore reversing a negative phenotype and must therefore be removing a negative factor [Fig. 4B(ii)]. Although these simple two factor models are appealing, there are a number of different TAR RNA-binding proteins that have been identified that may need to be incorporated into any model. Furthermore, the model as it stands requires us to propose that the interaction of Tat with its target that results in activation is transient. This proposal is in fact supported by observations that Tat works only on nascent RNA (4) and that Tat derivatives with enhanced RNA binding affinities are less active than wild-type Tat (42). A transient interaction would explain why CTAR2 can function to remove a negative cellular factor and so permit Tat activation yet it does not function as an inhibitor of activation by virtue of binding to Tat (this issue is discussed in detail in reference 7). Consistent with this interperetation, a candidate protein that binds specifically to the TAR bulge region has been identified in gel shift assays in both *Xenopus* oocyte and HeLa cell nuclear extracts (31a), and in in vitro binding assays, it has a far higher affinity for the bulge than Tat does. Irrespective of the details of the model, it is clear that in oocytes there are two independent conditions in which TAR-specific activation by Tat occurs independently of the loop. We could therefore use these assays to test whether inhibition by Ro24-7429 was dependent on the normal functions of the TAR loop sequences.

As shown in Figure 5a, Ro24-7429 at a concentration of 0.37 μM was inhibitory to Tat activation of a wild-type TAR reporter (38 RNA) (lanes 4 and 5), but it had little effect on activation of a loop mutant reporter (43 RNA) in the presence of excess CTAR2 competitor (lanes 2 and 3). This observation is independently repeated in Fig. 5b (lanes 5 and 6). Similarly, as shown in Fig. 5b, whereas wild-type TAR RNA is again completely inhibited by Ro24-7429 (lane 3 compared with lane 2), the drug had no effect on activation of the same reporter in the presence of excess CTAR2 and CTAR4 (compare lanes 10 and 13). The independent effects of these competitors are shown in lanes 8 and 9 to confirm that CTAR2 does not compete (lane 8) and CTAR4 does compete (lane 9) for Tat activation. In assay A (Fig. 4 and 5a), Tat activation must be independent of the classical loop-binding factors because of the nature of the mutation in the reporter. In assay B (Fig. 4 and 5b), it is likely from the arguments presented above that Tat activation is also occurring in the absence of the loop-binding proteins. Clearly, the ability of the drug to inhibit the specific Tat-TAR interaction is conditional. The simplest explanation based on these observations is that it is conditional upon the requirement for loop-binding proteins for Tat activation. One possibility is that the drug inhibits the binding of a loop-binding protein to TAR; however, one set of observations is inconsistent with this hypothesis. In lane 11 of Fig. 5b, we tested the effect of the drug on a wild-type TAR RNA in the presence of excess CTAR2. This competitor normally has no effect on Tat activation (lane 8), and the drug completely abolished activation. However, if CTAR2 has removed all of the putative negative factor as we have argued above, then Tat activation should be independent of any loop-binding proteins and would be resistant to the effects of any agent that blocked the binding of a protein to the loop. This is clearly not the case. We therefore suggest that Ro24-7429 functions by interacting with a loop-binding protein but that it exerts its effect on this protein only once the protein is bound to TAR.

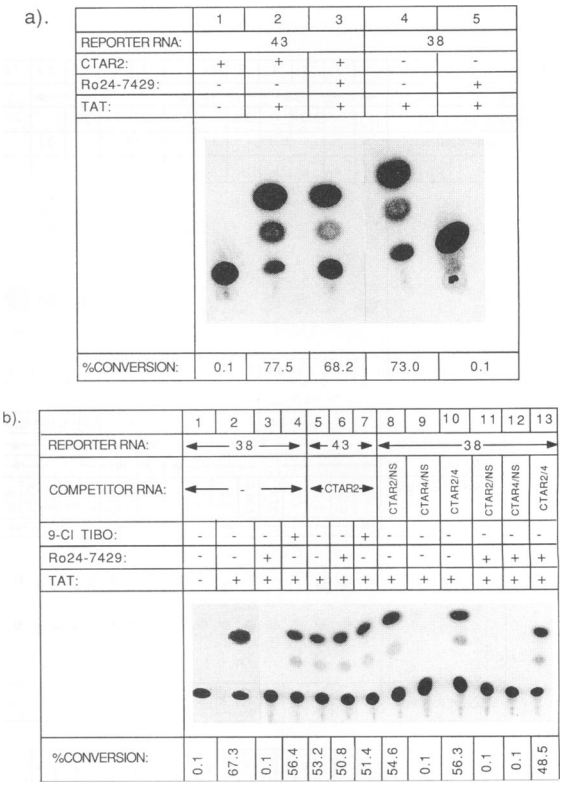

FIG. 5. Effect of Ro24-7429 on loop-independent Tat activation. (a) CAT assays to show the effect of 0.37 μM Ro24-7429 on Tat activation of a TAR loop mutant reporter RNA (43 RNA) in the presence of the competitor CTAR2 (lanes 1 to 3). This is the configuration shown in Fig. 4A(ii). The inhibitory effect on wild-type RNA (38 RNA) is also shown (lanes 4 and 5). (b) CAT assays to show the effect of 0.37 μM Ro24-7429 on Tat activation of a wild-type reporter RNA (38 RNA) in the presence of the competitors CTAR2 and CTAR4. This is the configuration shown in Fig. 4B(ii). CTAR2/NS and CTAR4/NS indicate an equal mix of the TAR-specific competitor and the nonspecific competitor; CTAR2/4 indicates an equal mix of CTAR2 and CTAR4. The specific competitors were present at a 125-fold molar excess. The effects of the single and double competitors on Tat activation are shown in lanes 8 to 10, and the effects of Ro24-7429 under these configurations are shown in lanes 11 to 13. The normal effects of benzodiazepines (Ro24-7429 and TIBO) on Tat activation of 38 RNA are shown for comparison (lanes 1 to 4). The effect of the drug in assay type A is also repeated (lanes 5 to 7).

Ro24-7429 does not abolish Tat binding to TAR RNA in vitro. Our data suggested strongly that Ro24-7429 was functioning by inhibiting the activity of TAR-binding cellular factors and not by blocking Tat binding to TAR. To confirm this, we tested the effect of Ro24-7429 on Tat binding in an in vitro binding assay. An in vitro Tat binding assay was established to give a Tat protein to probe ratio at which Tat was limiting and the binding was specific. As shown in Fig. 6, a mobility shift of TAR RNA was seen in the presence of Tat (compare lane 1 with lane 11). This shift was not observed with a TAR RNA probe that had a three-base substitution in the bulge sequence (lane 2), but a three-base substitution in the loop sequence had no effect (lane 3) on the mobility shift. Increasing concentrations of Ro24-7429 from 0.37 to 37 μM had no effect on Tat binding (lanes 7 to 9). The DMSO diluant also had no effect (lanes 4 to 6). These data show that Ro24-7429 does not affect the Tat-TAR interaction in vitro.

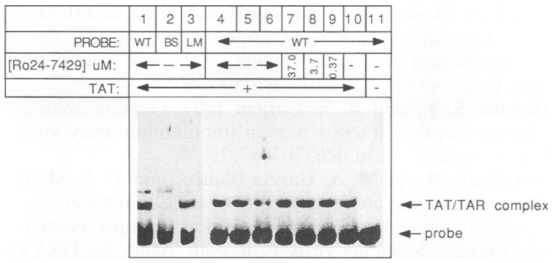

FIG. 6. Effect of Ro24-7429 on Tat binding to TAR in vitro. Tat was bound to radiolabelled TAR RNAs in vitro in the presence or absence of Ro24-7429, and the products were analyzed by gel mobility shift assay. WT, wild-type TAR RNA; BS, TAR RNA with substitution mutations in the bulge; LM, TAR RNA with substitution mutations in the loop. The TAR RNA probe and the retarded Tat-TAR complex are indicated.

This finding confirms recently reported unpublished data (cited in reference 23) and suggests that the in vivo mechanism of action of Ro24-7429 is not likely to be via a direct effect on Tat binding to TAR.

DISCUSSION

We have further characterized the antiviral activity of the benzodiazepine Ro24-7429. We have shown that although this compound has potent antiviral activity when measured early after infection, there is significant breakthrough of viral replication, and by 2 weeks, virus titers are as high as in untreated controls. The compound therefore appears to be only partially inhibitory for virus replication. Similar conclusions have been reached for the related compound Ro5-3335 in one analysis (49), although greater potency has also been reported for both compounds (23, 24). It appears that the potency of these drugs may be very dependent on the nature of the assay and the cell type that is used. Both compounds have a specific effect on TAR-dependent activation by Tat, although two groups (this report and reference 49) have indicated that the specificity index can be very low. A delay in viral replication has recently been reported for a virus that was specifically mutated to lack the Tat gene (15). This finding suggests that even if the drugs completely inhibited Tat action, their effects might be limited to delaying replication rather than to blocking it completely.

We have now demonstrated a marked effect of these compounds on a specific Tat-TAR interaction that leads to translational activation in *Xenopus* oocytes. In this system, the drugs are inhibitory in a dose-dependent fashion, and complete inhibition is observed at concentrations at which no general perturbation of translation is detectable. The simplest explanation for the fact that these compounds have an effect in two very different assays would be that they are interfering with Tat binding to TAR rather than with any effector mechanisms. However, we have provided genetic evidence that in *Xenopus* oocytes, this does not seem to be the case. Rather, it appears that the drugs function to inhibit the activity of a cellular factor that binds to the TAR loop sequence. This conclusion is based on the finding that in two separate assays for TAR-dependent, Tat-dependent activation in the absence of cellular loop-binding factors, the drug had no effect. Furthermore, in keeping with the conclusions of the genetic analysis, we were unable to detect any effect of Ro24-7429 on Tat binding to TAR in a specific in vitro binding biochemical assay.

At present, the precise role of loop-binding proteins is not known, but our study in oocytes indicates that they are not obligatory for Tat activation. This is also the finding in human cells, in which Tat activation can occur in the absence of TAR if Tat is located in the vicinity of the promoter either via a heterologous RNA-binding protein (42, 44) or via a DNA binding site (45). It has also been noted that TAR-dependent Tat activation in some rodent cells can occur independently of the loop sequences (22). It is possible, as we have argued for the *Xenopus* system (7), that a major function of the loop-binding factors is to facilitate access to TAR by Tat by preventing negative factors masking the Tat binding site. A significant control of the Tat activation process might then be achieved via the relative balance of the levels or activity of positive and negative cellular factors. Such an imbalance of cellular TAR-binding factors has been invoked to explain the inefficiency of Tat activation in some rodent cells (2).

We do not know how closely events in the oocyte parallel events in mammalian cells, although many of the molecular features of the Tat-TAR interaction in the two systems are the same (27). In the absence of a full biochemical characterization of TAR-binding factors, we cannot exclude the possibility that there are more complex interactions between Tat, TAR, and cellular factors in mammalian cells compared with *Xenopus* oocytes. It has, for example, been recently suggested that Tat binds to TAR only as a preformed complex with loop-binding cellular factors (28, 29), which is not easily reconciled with the data presented here for *Xenopus* oocytes. Our observations that Ro24-7429 and Ro5-3335 inhibit Tat activation in oocytes further strengthens the notion that there are at least some common mechanistic features, although it is formally possible that the drugs have entirely different modes of action in the different cell types.

ACKNOWLEDGMENTS

This research was supported by Glaxo and the MRC ADP. M.B. is a Royal Society research fellow.

We are grateful to Wolfgang Nacken for providing purified Tat protein.

REFERENCES

1. **Aldovini, A., and B. D. Walker.** 1990. Techniques in HIV research. Stockton Press, New York.
2. **Alonso, A., D. Derse, and M. B. Peterlin.** 1992. Human chromosome 12 is required for optimal interactions between Tat and TAR of human immunodeficiency virus type 1 in rodent cells. J. Virol. **66**:4617–4621.
3. **Bellemin, A. R., J. V. Earley, M.-C. Hsu, and S. Y.-K. Tam.** 1991. European patent application number 0462522A1.
4. **Berkhout, B., R. H. Silverman, and K.-T. Jaeng.** 1989. Tat transactivates the human immunodeficiency virus through a nascent RNA target. Cell **59**:273–282.
5. **Blanchard, A. D., R. Powell, M. Braddock, A. J. Kingsman, and S. M. Kingsman.** 1992. An adenosine at position 27 in the human immunodeficiency virus type 1 *trans*-activation response element is not critical for transcriptional or translational activation by Tat. J. Virol. **66**:6769–6772.
6. **Braddock, M., A. Chambers, W. Wilson, M. P. Esnouf, S. E. Adams, A. J. Kingsman, and S. M. Kingsman.** 1989. HIV-1 Tat "activates" presynthesised RNA in the nucleus. Cell **58**:269–279.
7. **Braddock, M., R. Powell, A. D. Blanchard, A. J. Kingsman, and S. M. Kingsman.** 1993. HIV-1 TAR RNA-binding proteins control Tat activation of translation in *Xenopus* oocytes. FASEB J. **7**:214–222.
8. **Braddock, M., A. M. Thorburn, A. Chambers, G. D. Elliott, G. J. Anderson, A. J. Kingsman, and S. M. Kingsman.** 1990. A nuclear translational block imposed by the HIV-1 U3 region is relieved by the Tat-TAR interaction. Cell **62**:1123–1133.
9. **Braddock, M., A. M. Thorburn, A. J. Kingsman, and S. M.**

Kingsman. 1991. Blocking of Tat-dependent HIV-1 RNA modification by an inhibitor of RNA polymerase II processivity. Nature (London) **350:**439–441.

10. **Calnan, B. J., S. Biancalana, D. Hudson, and A. D. Frankel.** 1991. Arginine-mediated RNA recognition: the arginine fork. Science **252:**1167–1171.

10a. **Cannon, P., and D. Wallace.** Unpublished data.

11. **Colvin, R. A., and M. A. Garcia-Blanco.** 1992. Unusual structure of the human immunodeficiency virus type 1 transactivation response element. J. Virol. **66:**930–935.

12. **Cullen, B. R.** 1991. Regulation of HIV-1 gene expression. FASEB J. **5:**2361–2368.

13. **Delling, U., L. S. Reid, R. W. Barnett, M. Y. X. Ma, S. Climie, M. Sumner-Smith, and N. Sonnenberg.** 1992. Conserved nucleotides in the TAR RNA stem of human immunodeficiency virus type 1 are critical for TAR binding and *trans*-activation: model for TAR RNA tertiary structure. J. Virol. **66:**3018–3025.

14. **Desai, K., P. M. Loewenstein, and M. Green.** 1991. Isolation of a cellular protein that binds to the human immunodeficiency virus Tat protein and can potentiate transactivation of the viral promoter. Proc. Natl. Acad. Sci. USA **88:**8875–8879.

15. **Dimitrov, D. S., R. Willey, H. Sato, L.-J. Chang, R. Blumenthal, and M. A. Martin.** 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. J. Virol. **67:**2182–2190.

16. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 Tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. EMBO J. **9:**4145–4153.

17. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, M. A. Skinner, and R. Valerio.** 1989. Human immunodeficiency virus 1 Tat protein binds trans-activation-responsive region (TAR) RNA in vitro. Proc. Natl. Acad. Sci. USA **86:**6925–6929.

18. **Feng, S., and E. C. Holland.** 1988. HIV-1 Tat trans-activation requires the loop sequences within TAR. Nature (London) **334:** 165–167.

19. **Gatignol, A., A. Buckler-White, B. Berkhout, and K.-T. Jeang.** 1991. Characterisation of a human TAR-RNA binding protein that activates the HIV-1 LTR. Science **251:**1597–1600.

20. **Gentz, R., C.-H. Chen, and C. A. Rosen.** 1989. Bioassay for *trans*-activation using purified human immunodeficiency virus Tat-encoded protein: *trans*-activation requires mRNA synthesis. Proc. Natl. Acad. Sci. USA **86:**821–824.

21. **Gunnery, S., S. R. Green, and M. B. Mathews.** 1992. Tat responsive region of human immunodeficiency virus type 1 stimulates protein synthesis in vivo and in vitro: relationship between structure and function. Proc. Natl. Acad. Sci. USA **89:**11556–11561.

22. **Hart, C. E., J. C. Galphin, M. A. Westhafer, and G. Schochetman.** 1993. TAR loop-dependent human immunodeficiency virus transactivation requires factors encoded on human chromosome 12. J. Virol. **67:**5020–5024.

23. **Hsu, M.-C., U. Dhingra, J. V. Early, M. Holly, D. Keith, C. M. Nalin, A. R. Richou, A. D. Schutt, S. Y. Tam, M. J. Potash, D. J. Volsky, and D. D. Richman.** 1993. Inhibition of type 1 human immunodeficiency virus replication by a Tat antagonist to which the virus remains sensitive after prolonged exposure in vitro. Proc. Natl. Acad. Sci. USA **90:**6395–6399.

24. **Hsu, M.-C., A. D. Schmutt, M. Holly, L. W. Slice, M. I. Sherman, D. D. Richman, M. J. Potash, and D. J. Volsky.** 1991. Inhibition of HIV replication in acute and chronic infections in vitro by a Tat antagonist. Science **254:**1799–1802.

25. **Johnston, R. F., S. C. Pickett, and D. L. Barker.** 1990. Autoradiography using storage phosphor technology. Electrophoresis **11:**353–360.

26. **Kim, S., R. Byrn, J. E. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.

27. **Kingsman, S. M., M. Braddock, and A. J. Kingsman.** 1991. An analysis of the Tat/TAR interaction in Xenopus oocytes, p. 221–237. *In* W. A. Haseltine and F. Wong-Staal (ed.), Genetic Structure and Regulation of HIV. Raven Press, New York.

28. **Luo, Y., S. Madore, T. J. Parslow, B. R. Cullen, and B. M. Peterlin.** 1993. Functional analysis of interactions between Tat and the *trans*-activation response element of human immunodeficiency virus type 1 in cells. J. Virol. **67:**5617–5622.

29. **Madore, S. J., and B. R. Cullen.** 1993. Genetic analysis of the cofactor requirement for human immunodeficiency virus type 1 Tat function. J. Virol. **67:**3703–3711.

30. **Marciniak, R. A., M. A. Garcia-Blanco, and P. A. Sharp.** 1990. Identification and characterisation of a HeLa nuclear protein that specifically binds to the trans-activation-response element of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87:**3624–3628.

31. **Masuda, T., and S. Harada.** 1993. Modulation of host cell nuclear proteins that bind to HIV-1 trans-activation-responsive element RNA by phorbol ester. Virology **192:**696–700.

31a. **Muckenthaler, M., A. Blanchard, M. Braddock, W. Nacken, A. J. Kingsman, and S. M. Kingsman.** Unpublished data.

32. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus trans-activator protein. Cell **48:**691–701.

33. **Nelbock, P., P. J. Dillon, A. Perkin, and C. A. Rosen.** 1990. A cDNA for a protein that interacts with the human immunodeficiency virus Tat transactivator. Science **248:**1650–1653.

34. **Ohana, B., P. A. Moore, S. M. Ruben, C. D. Southgate, M. R. Green, and C. A. Rosen.** 1993. The type 1 human immunodeficiency virus Tat binding protein is a transcriptional activator belonging to an additional family of evolutionarily conserved genes. Proc. Natl. Acad. Sci. USA **90:**138–142.

35. **Pauwels, R., K. Andries, J. Desmyter, D. Schols, M. J. Kukla, H. J. Breslin, A. Raeymaeckers, J. V. Gelder, R. Woestenborghs, J. Heykants, K. Schellekens, M. A. C. Janssen, E. DeClerq, and P. A. J. Janssen.** 1990. Potent and selective inhibition of HIV-1 replication in vitro by a novel series of TIBO derivatives. Nature (London) **343:**470–473.

36. **Puglisi, J. D., R. Tan, B. J. Calnan, A. D. Frankel, and J. R. Williamson.** 1992. Conformation of the TAR RNA arginine complex by NMR spectroscopy. Science **257:**76–80.

37. **Ratnasabapathy, R., M. Sheldon, L. Johal, and N. Hernandez.** 1990. The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAs from various mRNA and snRNA promoters. Genes Dev. **4:**2061–2074.

38. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of regulatory sequences in the human T-cell lymphotrophic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41:**813–823.

39. **Rounseville, M. P., and A. Kumar.** 1992. Binding of a host cell nuclear protein to the stem region of human immunodeficiency virus type 1 *trans*-activation-responsive RNA. J. Virol. **66:**1688–1694.

40. **Sanger, F. S., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

41. **Sarver, N., R. J. Black, S. Bridges, and L. Chrisey.** 1992. Frontiers in HIV-1 therapy: fourth conference of the NIAID national cooperative drug discovery groups-HIV. AIDS Res. Hum. Retroviruses **8:**659–666.

42. **Selby, M. J., and M. B. Peterlin.** 1990. Trans-activation by HIV-1 Tat via a heterologous RNA binding protein. Cell **62:**769–776.

43. **Sheline, C. T., L. H. Miloco, and K. A. Jones.** 1991. Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR RNA hairpin. Genes Dev. **5:**2508–2520.

44. **Southgate, C., M. L. Zapp, and M. R. Green.** 1990. Activation of transcription by HIV-1 Tat protein tethered to nascent RNA through another protein. Nature (London) **345:**640–642.

45. **Southgate, C. M., and M. R. Green.** 1991. The HIV-1 Tat protein activates transcription from an upstream DNA binding site: implications for Tat function. Genes Dev. **5:**2496–2507.

46. **Sullenger, B. A., H. F. Gallardo, G. E. Ungers, and E. Gilboa.** 1991. Analysis of *trans*-acting response decoy RNA-mediated inhibition of human immunodeficiency virus type 1 transactivation. J. Virol. **65:**6811–6816.

47. **Sumner-Smith, M., S. Roy, R. Barnett, L. S. Reid, R. Kuperman, U. Delling, and N. Sonnenberg.** 1991. Critical chemical features in

trans-acting responsive RNA are required for interaction with human immunodeficiency virus type 1 Tat protein. J. Virol. **65:**5196–5202.

48. **Weeks, K. M., C. Ampe, S. C. Schultz, T. A. Steitz, and D. M. Crothers.** 1990. Fragments of the HIV-1 Tat protein specifically bind TAR RNA. Science **249:**1281–1285.
49. **Witvrouw, M., R. Pauwels, A.-M. Vandamme, D. Schols, D. Reymen, N. Yamamoto, J. Desmyter, and E. de Clercq.** 1992. Cell type-specific anti-human immunodeficiency virus type 1 activity of the transactivation inhibitor Ro5-3335. Antimicrob. Agents Chemother. **36:**2628–2633.
50. **Wu, F., J. Garcia, D. Sigman, and R. Gaynor.** 1991. Tat regulates binding of the human immunodeficiency virus trans-activating region RNA loop-binding TRP-185. Genes Dev. **5:**2128–2140.