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The Human Immunodeficiency Virus Type 1 Tat Antagonist, Ro 5-3335, Predominantly Inhibits Transcription Initiation from the Viral Promoter

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Tat, the transcriptional transactivator protein of the human immunodeficiency virus type 1 (HIV-1), is required for viral replication in vitro. The Tat antagonist, Ro 5-3335, and its analog, Ro 24-7429, have been shown to inhibit replication of HIV-1 and to reduce steady-state viral RNA in infected cells (M.-C. Hsu et al., Science 254:1799–1802, 1991, and M.-C. Hsu et al., Proc. Natl. Acad. Sci. USA 90:6395–6399, 1993). Analysis of HIV-1 long terminal repeat-driven reporter gene transcription in a recombinant adenovirus by nuclear run-on assay indicated that the drug predominantly inhibits Tat-dependent initiation and also exerts a measurable effect on elongation. This result may imply a common mechanism for Tat-mediated transcription initiation and elongation.

The human immunodeficiency virus type 1 (HIV-1) regulatory protein, Tat, is required for viral replication in vitro (5–8, 30), suggesting that Tat could be a good target for therapeutic intervention. The major action of Tat is transcriptional (15, 20, 24, 25, 28), although posttranscriptional activity has been reported for some systems (2, 3, 29). Tat has been shown to facilitate transcription initiation and/or elongation, depending on the basal activity of the HIV-1 long terminal repeat (LTR) promoter, which varies in different cell lines and plasmid constructs (18, 21). Analysis of Tat function is further complicated by a second mode of activity of the HIV-1 LTR promoter which can initiate nonprocessive transcription, producing short transcripts of approximately 59 nucleotides (23, 32).

Ro 5-3335, a Tat antagonist identified by a random screen employing an HIV-1 LTR-driven indicator gene, and its analog, Ro 24-7429, have been shown to inhibit replication of HIV-1 in acute and chronically infected cells, to reduce steady-state viral RNA in chronically infected T lymphocytes, and to partially restore CD4 expression in infected T lymphocytes (10–12, 31). To elucidate the mechanism of action of these compounds, we analyzed the effect of Ro 5-3335 on transcription initiation and elongation, employing a recombinant adenovirus system developed in the laboratory of Michael B. Mathews (27, 28). In this system, a replication-defective adenovirus vector is used to introduce an HIV-1 promoter-directed chloramphenicol acetyltransferase (CAT) gene into cultured cells. Infection of HeLa cells constitutively expressing the HIV-1 *tat* gene resulted in specific transactivation of the HIV-1 CAT reporter over 100-fold of that seen in the parental HeLa cell line (27, 28). Efficient infection by the recombinant adenovirus facilitated an analysis of run-on transcripts from isolated nuclei of infected cells. The results revealed a difference in the rates of transcription proximal and distal to the promoter and indicated that Tat not only acts to stimulate the initiation of transcription from the HIV-1 promoter but also stabilizes elongation in this system (20, 21).

The specificity of Ro 5-3335 for Tat-mediated transactivation was confirmed in the recombinant adenovirus system. HeLa and HeLa-*tat* cells were infected with HIV-1CATad, a replication-defective adenovirus containing an HIV-1 LTR CAT reporter gene (20), at a multiplicity of infection of 50 PFU per cell and treated with Ro 5-3335 at 2 h postinfection. The cells were allowed to incubate for 40 to 46 h before harvesting. Standard CAT assays were performed on equivalent amounts of extracted protein as determined by the Bio-Rad (Hercules, Calif.) protein assay. Whereas Ro 5-3335 had little effect on HIV-1 CAT expression in HeLa cells (Fig. 1A), increasing concentrations of the drug inhibited CAT expression in HeLa-*tat* cells in a dose-dependent manner (Fig. 1B). Incubation with 25 to 50 μM Ro 5-3335 resulted in an 85 to 90% inhibition of CAT activity (Fig. 1B, lanes 7 to 12). Thus, Ro 5-3335 inhibits HIV-1 LTR promoter-driven gene expression in the recombinant adenovirus system when the Tat transactivator protein is present, but it is not inhibitory in its absence.

The drug's effect on a heterologous promoter in this system was also examined by using the recombinant adenovirus, RSV-CATad, which contains a CAT reporter gene under the control of the Rous sarcoma virus LTR (27, 28). Ro 5-3335 had no significant effect on Rous sarcoma virus LTR-driven CAT gene expression in HeLa or HeLa-*tat* cells (data not shown), indicating that the drug acts specifically on the HIV-1 LTR. Furthermore, Ro 5-3335 had no significant effect on an HIV-1 CAT recombinant adenovirus in which +26 to +41 of the TAR region had been deleted (HIV-1CATadBH [20]) in HeLa-*tat* cells (data not shown). These data confirm the specificity of Ro 5-3335 for HIV-1 LTR-directed gene expression in the presence of Tat. They also indicate that the drug does not inhibit any process specific to the recombinant adenovirus.

We next analyzed the drug's effects on steady-state levels of RNA. Cells were infected with the recombinant adenovirus and incubated in the presence of Ro 5-3335 under the same conditions as those used for the CAT assays. Poly(A)$^+$ RNA was isolated by using the Micro-Fast Track mRNA isolation kit (Invitrogen, San Diego, Calif.) and subjected to Northern (RNA) analysis. Hybridization with a 750-bp probe representing the CAT gene resulted in the detection of three CAT-specific bands ranging in size from approximately 1,500 to 2,900 nucleotides (Fig. 2). All three bands were responsive to Ro 5-3335 in a dose-dependent manner, suggesting that all

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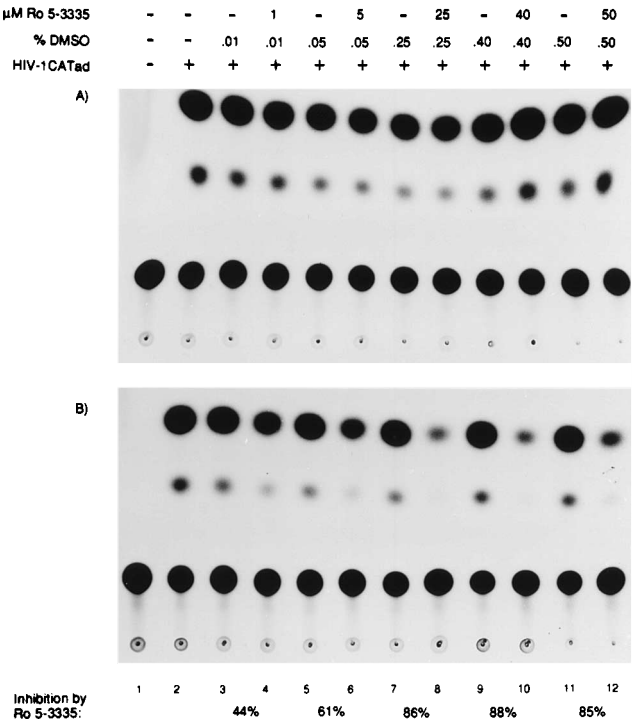

FIG. 1. Effects of Ro 5-3335 on HIV-1 CAT expression in HeLa and HeLa-*tat* cells. (A) HeLa cells were infected with HIV-1CATad and treated with increasing concentrations of Ro 5-3335. Control cells were treated with the corresponding concentration of the drug diluent, DMSO. The activity of 100 μg of cell extract was assayed after a 5-h incubation. (B) HeLa-*tat* cells were infected and treated with the drug as described for panel A. The activity of 1 μg of cell extract was assayed after a 5-min incubation. Percent inhibitions of CAT activity by Ro 5-3335 were calculated by comparing the percent acetylation with drug to the percent acetylation with the corresponding DMSO control. The data displayed are averages of three assays, except for the 1 and 40 μM treatments, which are the averages of two assays. Recombinant adenoviruses and cell lines were generous gifts from Michael Laspia.

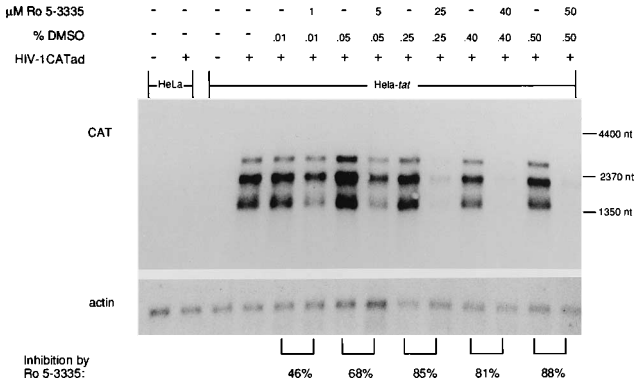

FIG. 2. Analysis of steady-state HIV-1 CAT RNA in the presence of Ro 5-3335. Northern blot analysis was performed on RNA isolated from drug-treated HIV-1CATad-infected HeLa and HeLa-*tat* cells (multiplicity of infection, 50 PFU per cell). RNA was hybridized to a 750-bp CAT probe and a 2-kb β-actin probe for control of RNA recovery. Size markers determined from a similar gel are depicted on the right. Percent inhibitions of RNA were calculated after normalization to the actin signal. The data displayed are averages of four assays, except for the 1 and 40 μM treatments, which are the averages of three assays.

three transcripts were directed by the HIV-1 promoter. Primer extension analysis of these transcripts using a primer corresponding to +30 to +60 of the CAT gene indicated that all three transcripts have the same 5′ end and are initiated at the HIV-1 LTR promoter (data not shown). We attributed the presence of the three bands to additional splice and/or polyadenylation sites in the adenovirus vector. The percent decreases in steady-state RNA were similar to the percent inhibitions of CAT activity at the corresponding drug concentrations (compare Fig. 1B and 2). The expression of Tat in the HeLa-*tat* cells, controlled by the simian virus 40 promoter (34), was not affected by the drug (Northern blot analysis; data not shown). Therefore, Ro 5-3335 exerted its inhibitory effect by decreasing the amount of Tat-mediated HIV-1 promoter-directed RNA.

In order to elucidate the underlying mechanism of action of Ro 5-3335 on Tat-mediated transcription, the nuclear run-on assay was employed. HeLa and HeLa-*tat* cells were infected with HIV-1CATad and then treated with 30 μM Ro 5-3335 or 0.3% dimethyl sulfoxide (DMSO) as a control. Nuclei were isolated 40 to 46 h following infection, and nuclear run-on assays were performed according to standard procedure (35). To normalize the hybridization efficiency, labelled RNA was fragmented with 0.2 N NaOH and hybridized to single-stranded DNA probes specifying distinct regions of the CAT gene template.

The probes used in this analysis, gifts from Michael Laspia, were antisense M13 single-stranded DNA probes which have previously been described (20, 21). Their respective positions along the HIV-1 CAT transcript are depicted in Fig. 3. In addition, a single-stranded M13 probe specifying the β-actin gene was used as a control for transcript recovery. The results of a representative nuclear run-on assay are shown in Fig. 4, and the relative transcription rate in each fragment for this experiment is listed in Table 1. The data are standardized to the β-actin signal and normalized to the uridine content of each RNA fragment.

As expected, transcription of the HIV-1 CAT template in HeLa cells in the absence of Tat was very low and a polarity effect was evident (Fig. 4, row 1; Table 1). Transcription was greatest in fragment I and dropped considerably (about 30-fold) along the length of the template. Incubation of these cells for 42 h in 30 μM Ro 5-3335 elicited no significant effect on transcription (Fig. 4, row 2; Table 1), confirming the CAT assay data.

Transcription of the HIV-1 CAT template in HeLa-*tat* cells was much more efficient than that seen in the parental HeLa line (Fig. 4, row 3; Table 1). The presence of Tat increased transcription in fragment I 7.7-fold and increased transcription in fragment VI 44-fold. Thus, Tat significantly increased promoter-proximal transcription and greatly reduced the magnitude of the polarity effect, confirming previous reports indicat-

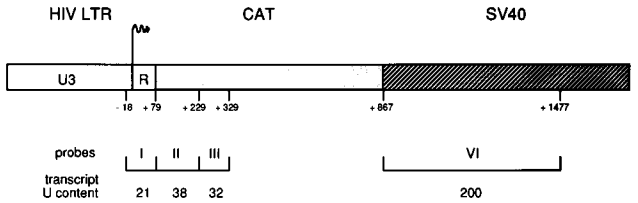

FIG. 3. Schematic of the DNA probes used in the nuclear run-on assay and their relative positions within the HIV-1 CAT transcription unit (20, 21). HIV-1CATad contains HIV-1 sequence −642 to +83 fused to the CAT gene and the simian virus 40 polyadenylation signal. Probes consisted of DNA fragments inserted into M13. Probe I extends from −18 to +83, probe II extends from +78 to +228, probe III extends from +229 to +333, and probe VI extends from +867 to +1476. The corresponding fragment uridine content is depicted under each probe. SV40, simian virus 40.

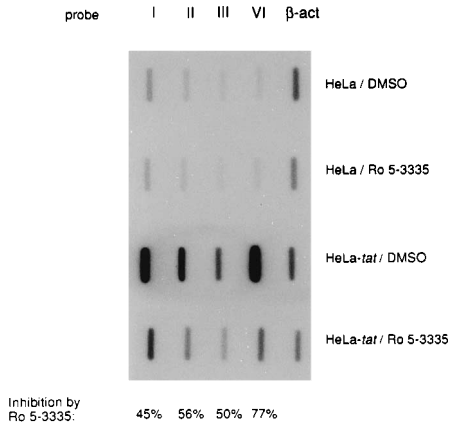

FIG. 4. Nuclear run-on transcription assay in the presence of the Tat antagonist, Ro 5-3335. Hybridization to the single-stranded M13 probes depicted in Fig. 3 along with a β-actin control for RNA recovery are shown. Nuclei were harvested, and run-on transcription assays were performed for 5 min at 30°C. The percent inhibitions below the blot are averages of six assays representing three independent preparations of recombinant adenovirus-infected nuclei.

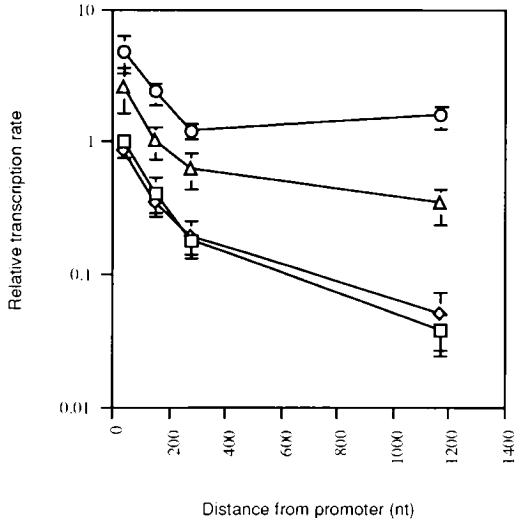

FIG. 5. Relative transcription rate throughout the HIV-1 CAT transcription unit. Nuclear run-on assays were quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Radioactivity hybridized to each probe was normalized to the β-actin signal and corrected for the transcript uridine content. The transcription within each fragment relative to the transcription in fragment I in HeLa cells incubated with 0.3% DMSO is plotted against the distance from the promoter midway through each fragment. The data are averages of six assays representing three independent preparations of recombinant adenovirus-infected nuclei. □, HeLa-DMSO control; ◇, HeLa-Ro 5-3335; ○, HeLa-*tat*-DMSO control; △, HeLa-*tat*-Ro 5-3335; nt, nucleotides.

ing effects on both transcription initiation and elongation (20, 21). Incubation of HeLa-*tat* cells for 42 h in the presence of 30 μM Ro 5-3335 decreased transcription significantly at each point along the length of the template (Fig. 4, row 4; Table 1). The magnitudes of inhibition of transcription by Ro 5-3335 within fragments I, II, and III were comparable (approximately 50%). The inhibition at fragment VI, representing the 3′-most point on the template assayed, however, was reproducibly higher (77%) (Fig. 4).

Relative transcription rate data from six nuclear run-on assays, representing three independent preparations of recombinant adenovirus-infected nuclei, were averaged and plotted against distance from the promoter (Fig. 5). The ability of Tat to increase initiation, as evidenced by the increase in the rate of transcription proximal to the promoter (probe I), and its ability to stabilize elongation, as evidenced by the greater increase in the rate of transcription at probe VI than at probe I, confirm previous data (20, 21). The rate of transcription proximal to the promoter was reduced in the presence of Ro 5-3335, as was the rate of transcription distal to the promoter, indicating an effect on Tat-mediated initiation. Furthermore, in the presence of the drug, the curve between probes III and VI was reproducibly shifted closer to that seen in the absence of Tat, indicating an inhibition of Tat's effect on elongation.

Conflicting data concerning the effect of Tat on transcription initiation and/or elongation from the HIV-1 LTR promoter have been reported (14, 15, 17, 19–21, 25, 33). It is now clear that the basal activity of the promoter in each system can apparently influence the function of Tat (18, 21). The absence of an effect on transcription initiation by Tat in studies of in vitro cell-free transcription could be a consequence of high basal promoter activity or inefficient reinitiation (17, 22, 24, 25). The low basal activity of the HIV-1 promoter in the recombinant adenovirus is reminiscent of an HIV-1 virus defective in Tat expression (30). Furthermore, the observed short transcripts initiated from nonprocessive Tat-independent transcription from the HIV-1 LTR might obscure Tat's effect on transcription initiation (19, 23, 26, 32). The apparent Tat-dependent stabilization of elongation may then be the result of the initiation of more processive transcription complexes (4, 14, 33). The observation that Ro 5-3335 predominantly inhibits Tat-dependent initiation and has a measurable effect on elongation, as determined by the run-on assay, suggests that there may indeed be a common mechanism for what seems like two separate activities. Recent reports showed that Tat can bind to a kinase (9), TFIID (16), and Sp1 (13). Whether any of the Tat-associated proteins is a target of Ro 5-3335 will be the subject of future investigation.

The clinical trials of Ro 24-7429 were halted due to side effects (the drug is a derivative of benzodiazepine, which exhibits central nervous system effects) in patients before antiviral activity could be demonstrated. One factor contributing to the lack of antiviral activity in patients may have been the extensive binding of the drug to human plasma proteins (99.7%; unpublished data), possibly decreasing drug availability to the nuclei. Recently, the intracellular concentration of the drug in the presence of 100% human serum was found to be 2 to 5% of that when 10% fetal bovine serum was used in in vitro studies (22a).

Tat remains an attractive molecular target for AIDS ther-

TABLE 1. Relative transcription rates in the HIV-1 LTR-directed transcription unit

Fragment	Relative transcription rate[a]			
	HeLa		HeLa-*tat*	
	DMSO control	Ro 5-3335	DMSO control	Ro 5-3335
I	1.0	0.96	7.7	3.6
II	0.25	0.30	2.1	1.0
III	0.18	0.19	1.4	0.81
VI	0.03	0.05	1.2	0.29

[a] Quantitation of results of the nuclear run-on assay shown in Fig. 4. The signal was quantified by PhosphorImager (Molecular Dynamics), corrected for the transcript uridine content, and normalized to the β-actin signal. Transcription is expressed relative to the transcription in fragment I in HeLa cells, treated with the drug diluent, DMSO.

apy. Inhibition of Tat has the potential to rescue T cells already infected with HIV-1, whereas an inhibitor of reverse transcriptase or protease could only prevent new rounds of infection. Continued effort in search of a better Tat antagonist might offer a more effective treatment in combination with other viral enzyme inhibitors or other known inhibitors of the viral promoter (e.g., pentoxyfylline, an inhibitor of tumor necrosis factor alpha [1]).

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