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Synergy between HIV-1 Tat and adenovirus E1A is principally due to stabilization of transcriptional elongation

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We studied the combined effects of Tat and general *trans*-activators, such as E1A and phorbol esters, on human immunodeficiency virus-1 (HIV-1) gene expression. Interaction between these two types of *trans*-activators may be involved in the transition from transcriptional quiesence during viral latency to active gene expression during productive infection. E1A cooperated with Tat to produce a fourfold greater increase in accumulation of full-length, cytoplasmic HIV-1-directed RNA than is expected if they were acting additively to increase RNA accumulation. Similarily, phorbol 12-myristate 13-acetate (PMA) also cooperated with Tat to elevate HIV RNA levels synergistically. Analysis of transcription rates across the HIV-1-directed transcription unit indicated, unexpectedly, that synergy between Tat and E1A could not be accounted for by increased promoter proximal transcription rates that were merely additive. However, Tat and E1A produced a greater than additive increase in transcription rates in the 3′ end of the gene. These findings imply that synergy between Tat and E1A (or other general transcriptional activators) is due principally to stabilization of transcriptional elongation. Furthermore, the observation that Tat elicits only a small increase in promoter proximal transcription in the presence of E1A suggests that the magnitude of the effect of Tat on initiation is decreased when the basal level of transcription is increased. These findings underscore the importance of the ability of Tat to stabilize elongation, as well as to stimulate initiation, in an HIV-1-directed transcription unit.

[*Key Words*: HIV; Tat; *trans*-activation; synergy; transcriptional initiation; transcriptional elongation]

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The *tat* gene of human immunodeficiency virus-1 (HIV-1) encodes a potent, essential *trans*-activator that greatly stimulates the expression of genes linked to the viral long terminal repeat (LTR) (Arya et al. 1985; Sodroski et al. 1985b). A sequence called *trans*-activation response (TAR) (Rosen et al. 1985), located downstream of the site of transcription initiation, minimally from nucleotides +14 to +44 relative to the transcription start (Hauber and Cullen 1988; Jakobovits et al. 1988), is required for Tat *trans*-activation of LTR-directed gene expression.

The RNA leader made by transcription of the TAR element is capable of forming an imperfect stem–loop structure (Muesing et al. 1987), and mutations that disrupt this structure greatly reduce *trans*-activation by Tat (Feng and Holland 1988). The introduction of flanking sequences that prevent the formation of TAR RNA secondary structure also prevents *trans*-activation (Berkhout et al. 1989), suggesting that Tat interacts with TAR at the RNA level. In support of this conclusion, a fusion protein in which Tat is linked to the RNA-binding domain of the Rev protein is capable of *trans*-activating a modified HIV-1 promoter where TAR has been replaced by the sequences that form the RNA-binding site for Rev (Southgate et al. 1990). This result indicates that the primary function of TAR is to provide a binding site for Tat at the HIV-1 promoter. Tat binds to the stem of TAR RNA in vitro (Dingwall et al. 1989), and mutations in the upper portion of the stem that prevent binding also reduce *trans*-activation greatly in vivo (Roy et al. 1990). Therefore, at least one component of Tat *trans*-activation requires that Tat bind to nascent TAR RNA. Cellular proteins are also likely to be involved in the interaction between Tat and TAR (Marciniak et al. 1990) because mutations in the loop region that do not affect Tat binding greatly reduce *trans*-activation (Roy et al. 1990; Weeks et al. 1990).

Regulation of HIV-1 gene expression by Tat has been proposed to occur at a number of different levels (for review, see Varmus 1988; Sharp and Marciniak 1989; Pavlakis and Felber 1990). Tat produces a large stimulation of HIV-1-promoted RNA levels (Cullen 1986; Peterlin et al. 1986; Wright et al. 1986; Hauber et al. 1987; Muesing et al. 1987; Rice and Mathews 1988a) so the predominant mode of regulation appears to be transcriptional (Hauber et al. 1987; Kao et al. 1987; Jakobovits et al. 1988; Jeang et al. 1988; Rice and Mathews 1988a; Sadaie et al. 1988; Laspia et al. 1989), but evidence for post-transcriptional regulation also exists (Cullen 1986; Fein-

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berg et al. 1986; Rosen et al. 1986; Wright et al. 1986; Braddock et al. 1989, 1990). In a model system consisting of replicating plasmids in COS cells, Kao et al. (1987) found that Tat does not increase the initiation of transcription but act to stimulate transcriptional elongation. They concluded that Tat acts as an antiterminator, relieving a block to transcriptional elongation occurring within the HIV-1 leader. On the other hand, our studies indicated that Tat acts through TAR to stimulate transcription in a bimodal manner: It increases transcriptional initiation and also stabilizes transcriptional elongation (Laspia et al. 1989). We compared *trans*-activation by Tat to *trans*-activation by the adenovirus E1A protein in HeLa cells infected with a recombinant adenovirus containing an HIV-1 LTR-promoted reporter gene. In the absence of *trans*-activators, the basal level of HIV-1-directed transcription is low and exhibits a marked polarity with RNA polymerase density, declining with increasing distance from the promoter. Tat both increases RNA polymerase density in the immediate vicinity of the promoter and reduces transcriptional polarity, whereas E1A increases polymerase density near the promoter without any detectable suppression of polarity.

The interaction between Tat and other transcriptional activators may be involved in the transition from low-level basal transcription early in infection or during viral latency to high-level expression in the active stages of viral growth (Cullen and Greene 1989; Pomerantz et al. 1990). Therefore, we have extended our analysis of HIV-1 gene regulation by examining the combined effect of Tat and the general transcriptional activator E1A on LTR-directed gene expression. Together, Tat and E1A produced a synergistic stimulation of LTR-promoted gene expression. The primary basis for the synergy was increased transcription in the 3′ end of the gene, implying that synergy is principally due to reduced elongational polarity rather than to increased transcriptional initiation. Studies with phorbol esters suggest that this is a general mechanism. In addition, we found that the magnitude of the stimulation of transcription initiation by Tat varies as a function of the basal level of transcription initiation. This observation may account for the apparent lack of a stimulatory effect of Tat on transcriptional initiation in some systems.

Results

Synergistic trans-*activation of LTR-promoted gene expression*

We examined the interaction between Tat and a general transcriptional activator, E1A, by analyzing reporter gene expression in HeLa cells infected with a recombinant adenovirus. The recombinant adenovirus HIV-1CATad contains HIV-1 LTR sequences −454 to +83 fused to the reporter gene chloramphenicol acetyltransferase (CAT) and SV40 processing signals (Fig. 2B, below; Rice and Mathews 1988a,b). This virus possesses the TAR element, including the sequences from +14 to +44 that confer Tat responsivity. In the experiments described here, Tat was expressed constitutively in HeLa cells from an integrated Tat cDNA under the control of the SV40 early promoter (HeLa/*tat*; Valerie et al. 1988), and E1A was supplied by coinfection with phenotypically wild-type adenovirus (Rice and Mathews 1988b).

In the experiment shown in Figure 1, Tat and E1A individually stimulated LTR-promoted CAT activity 371-fold and 21-fold, respectively. In cells that expressed Tat and E1A simultaneously, however, LTR-directed CAT activity was stimulated 1772-fold, which is more than four times that expected if they acted additively. Therefore, these two *trans*-activators are able to cooperate to produce synergistic stimulation of LTR-promoted gene expression.

Synergy in the accumulation of LTR-directed RNA

To elucidate the nature of this synergy, we first examined the combined effects of Tat and E1A on LTR-promoted RNA levels. Two classes of HIV-1-promoted RNA are detected by RNase protection assay (Fig. 2A): a full-length poly(A)$^+$ class that protects probe fragments of ~83 nucleotides and a short poly(A)$^-$ class that protects probe fragments of 55 and 59 nucleotides and represents terminated or processed RNA (Fig. 2B). The protected species of intermediate lengths have been ascribed to partial degradation of full-length RNA in the protection assay because they are also observed with poly(A)$^+$ RNA (Laspia et al. 1989).

Quantitation of the 83-nucleotide protected fragment indicated that Tat and E1A individually stimulated the level of full-length transcript 16-fold and 4-fold, respec-

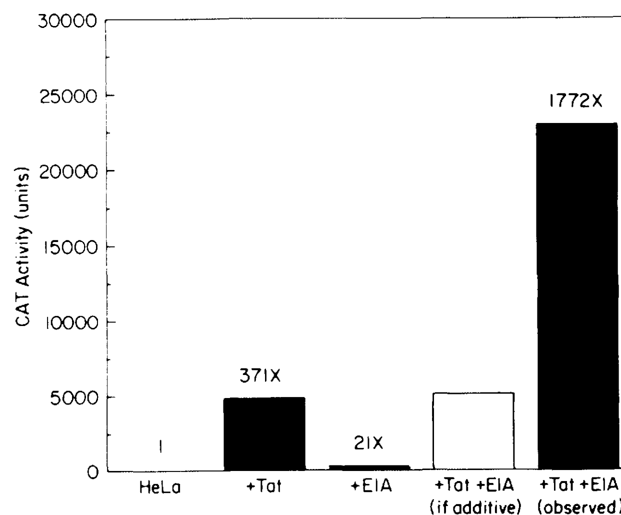

Figure 1. Expression of CAT in recombinant adenovirus-infected cells. CAT assays were performed on lysates from HIV-1CATad-infected HeLa cells (HeLa), HeLa/*tat* cells (+Tat), HeLa cells coinfected with *dl*309 (+E1A), or HeLa/*tat* cells coinfected with *dl*309 (+Tat +E1A). HIV-1CATad infections were carried out at an moi of 100, and *dl*309 coinfections were carried out at an moi of 50. Cells were harvested 24 hr postinfection. Stimulation relative to HeLa is shown above each bar. The data presented are the average of three experiments.

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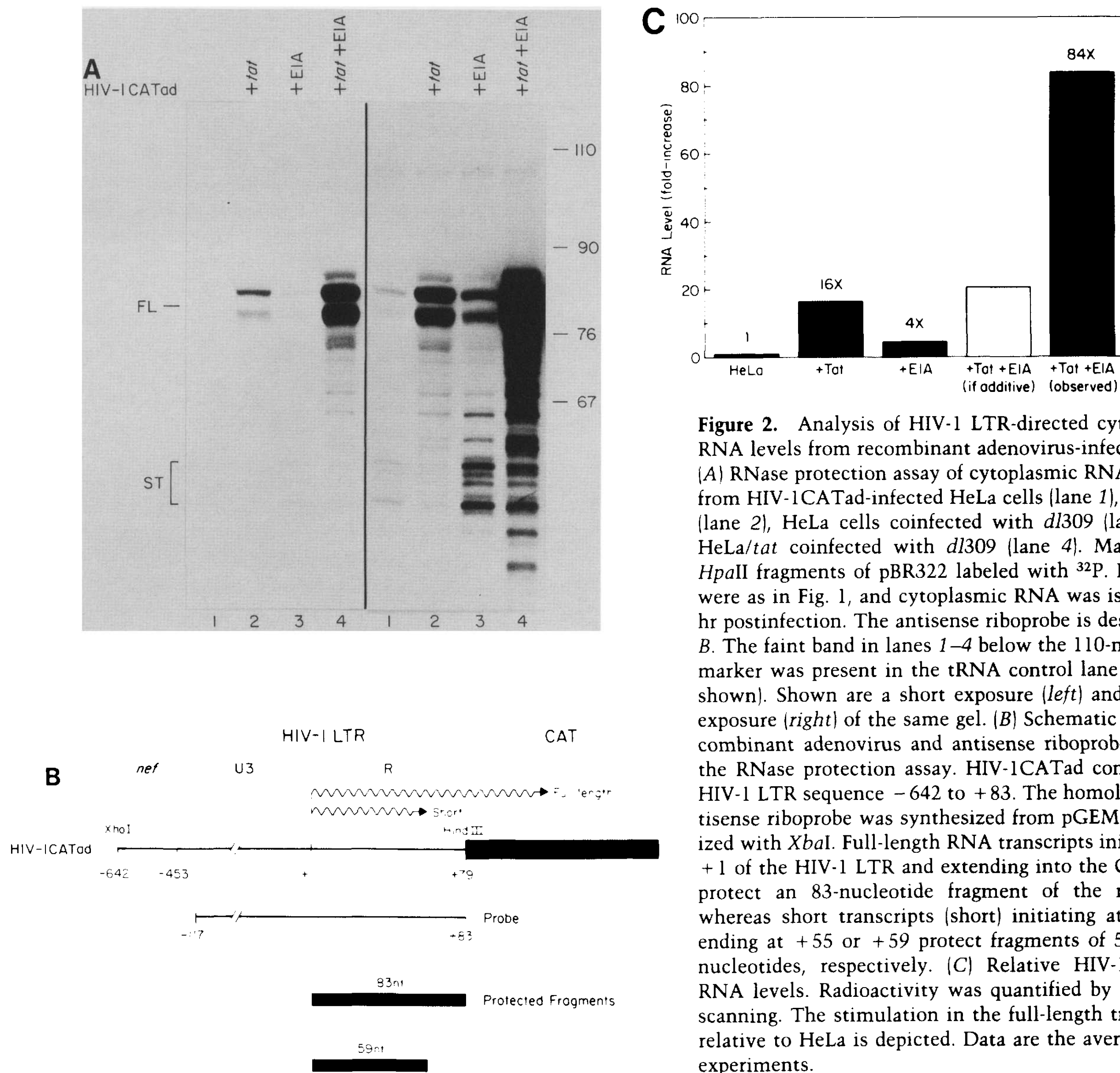

Figure 2. Analysis of HIV-1 LTR-directed cytoplasmic RNA levels from recombinant adenovirus-infected cells. (*A*) RNase protection assay of cytoplasmic RNA isolated from HIV-1CATad-infected HeLa cells (lane *1*), HeLa/*tat* (lane *2*), HeLa cells coinfected with *dl*309 (lane *3*), or HeLa/*tat* coinfected with *dl*309 (lane *4*). Markers are *Hpa*II fragments of pBR322 labeled with ^{32}P. Infections were as in Fig. 1, and cytoplasmic RNA was isolated 24 hr postinfection. The antisense riboprobe is described in *B*. The faint band in lanes *1–4* below the 110-nucleotide marker was present in the tRNA control lane (data not shown). Shown are a short exposure (*left*) and a longer exposure (*right*) of the same gel. (*B*) Schematic of the recombinant adenovirus and antisense riboprobe used in the RNase protection assay. HIV-1CATad contains the HIV-1 LTR sequence −642 to +83. The homologous antisense riboprobe was synthesized from pGEM23 linearized with *Xba*I. Full-length RNA transcripts initiating at +1 of the HIV-1 LTR and extending into the CAT gene protect an 83-nucleotide fragment of the riboprobe, whereas short transcripts (short) initiating at +1 and ending at +55 or +59 protect fragments of 55 and 59 nucleotides, respectively. (*C*) Relative HIV-1-directed RNA levels. Radioactivity was quantified by direct gel scanning. The stimulation in the full-length transcripts relative to HeLa is depicted. Data are the average of six experiments.

tively (Fig. 2A, lanes 2 and 3; Fig. 2C). Simultaneous expression of Tat and E1A produced a large increase (84-fold) in the amount of LTR-directed full-length RNA (Fig. 2A, lane 4). This increase, again, was over four times that predicted if they acted additively (Fig. 2C), indicating that Tat and E1A cooperate to produce a synergistic stimulation in the accumulation of full-length LTR-promoted RNA. As expected, Tat *trans*-activation and synergy between Tat and E1A was abolished by a deletion in the TAR region (data not shown). A similar synergistic stimulation of LTR-directed CAT enzyme and full-length RNA levels was observed as early as 8 hr postinfection (data not shown), as well as at the 24-hr time point shown.

Tat produced a slight reduction in the level of short transcripts in Figure 2A, although the short transcripts remained unchanged in other experiments (Laspia et al. 1989, and data not shown). As shown previously, E1A increased the level of the short transcripts. The simultaneous expression of Tat and E1A caused an increase in the accumulation of short transcripts, but this increase was not synergistic. In the presence of Tat + E1A, the level of short transcripts was slightly lower than in the presence of E1A alone but higher than in the presence of Tat alone. These observations suggest that when Tat and E1A act in concert, each affects the elevated level of transcription produced by the other in a fashion similar to their individual effects on basal transcription. Therefore, it seems that synergy may be due to the combined independent actions of Tat and E1A rather than to a novel stimulatory mechanism.

CAT RNA utilization correlates with RNA accumulation

Noting a greater increase in the accumulation of CAT

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activity than in CAT RNA in the presence of Tat, several workers have concluded that Tat can stimulate gene expression at the post-transcriptional level (for review, see Sharp and Marciniak 1989; Pavlakis and Felber 1990). This idea remains controversial as other workers have failed to detect such an effect. Comparison of the data presented in Figures 1 and 2C revealed a substantially greater increase in CAT activity than in full-length CAT RNA in the presence of Tat alone or Tat + E1A. This discrepancy was not Tat-specific, however, as an increase was also seen with E1A alone. Figure 3 shows how CAT RNA utilization, measured as the ratio of CAT enzyme to CAT RNA, varied with the level of CAT RNA present in cells expressing either E1A, Tat, or Tat + E1A. RNA utilization increased with increasing RNA level until a plateau was reached. This correlation implies that up to a limit, each RNA molecule gives rise to more CAT enzyme at high RNA concentration than at low RNA concentration, regardless of the presence or absence of Tat. This interpretation is consistent with previous data indicating that Tat has no direct effect on CAT RNA translation (Rice and Mathews 1988a). Another interpretation, which in our view is less likely, is that Tat and E1A act separately to increase the utilization of TAR CAT mRNA. While the mechanism for the effect is unknown, it could be translational, perhaps related to the interactions of TAR RNA with the protein kinase DAI (Edery et al. 1989; Sen-Gupta and Silverman 1989; Gunnery et al. 1990; Sen-Gupta et al. 1990). However, post-translational mechanisms, such as concentration-dependent changes in CAT enzyme stability, also cannot be excluded.

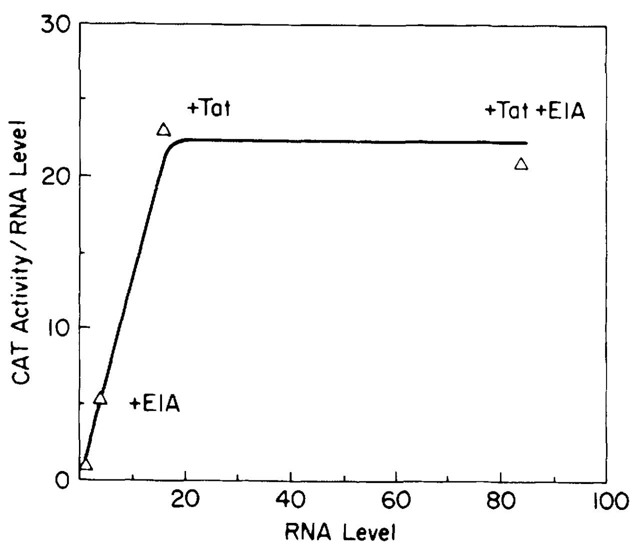

Figure 3. Accumulation of CAT activity at different cytoplasmic RNA levels. The efficiency of RNA utilization is defined as the ratio of stimulation of CAT activity to the stimulation of full-length RNA level. This parameter is plotted against the stimulation in full-length RNA for E1A, Tat, or Tat + E1A. The data are from Figs. 1 and 2C.

Synergy is not due to increased transcriptional initiation

To pursue further the mechanism underlying the synergy between Tat and E1A on RNA accumulation, we studied their combined effects on HIV-1-promoted transcription rates by nuclear run-on analysis (Greenberg and Ziff 1984). Nuclei were isolated from recombinant adenovirus-infected cells, and nascent transcripts were pulse-labeled with [α-^{32}P]UTP. The RNA was isolated and hybridized to short single-stranded DNA probes corresponding to the HIV-1 leader (fragment I) and to the 5′ end of the CAT gene (fragments II and III; Fig. 4B). Under these conditions, the amount of radioactivity hybridizing to each antisense (+) probe is a measure of the relative transcription rate in that region of the gene and provides an estimate of RNA polymerase distribution (Laspia et al. 1989).

In the absence of *trans*-activators, as we found earlier, transcription was low in the promoter proximal region (fragment I) and decreased markedly in the CAT sequences (fragments II and III) (Fig. 4A, HeLa). Both Tat and E1A increased transcription in fragment I (Fig. 4A), as well as a subfragment of I, fragment IA, which correspond to the first 24 nucleotides of the HIV-1 leader (Fig. 4C). This suggests that both Tat and E1A increase RNA polymerase density in the immediate vicinity of the promoter, consistent with them both acting to increase transcriptional initiation (Laspia et al. 1989). Although the formal possibility exists that Tat might overcome a block to elongation within the first several nucleotides, giving the appearance of an increase in initiation, no evidence exists to support this. Indeed, were there a complete block to elongation near the transcription start site we would not expect E1A to increase promoter proximal transcription.

As shown earlier, Tat also suppressed the polar effect on transcription in the CAT sequences, whereas E1A did not. Unexpectedly, simultaneous expression of Tat and E1A did not elicit any obvious increase in transcription in fragment I relative to either Tat or E1A alone, although quantitation of subsequent experiments revealed a nearly additive effect (see below). Furthermore, Tat + E1A did not produce a greater-than-additive increase in transcription rates in fragment IA (Fig. 4C). Our inability to observe a synergistic increase in promoter proximal transcription was not due to saturation of the DNA probes bound to the filters, as a threefold dilution of the labeled nascent RNA was reflected in a threefold decrease in signal (Fig. 4C). Therefore, increased initiation cannot account for the synergy produced by Tat + E1A on HIV-1-directed RNA levels.

In the adjacent region of the CAT gene (fragments II and III), Tat + E1A again did not elicit any obvious increase in transcription rates over Tat alone (Fig. 4), so synergy also cannot be explained by a suppression of transcriptional polarity that can be detected over the first 333 nucleotides of the LTR-promoted CAT transcription unit. However, Tat + E1A produced an increase over E1A alone, indicating that when transcriptional initiation is elevated by E1A, Tat suppresses tran-

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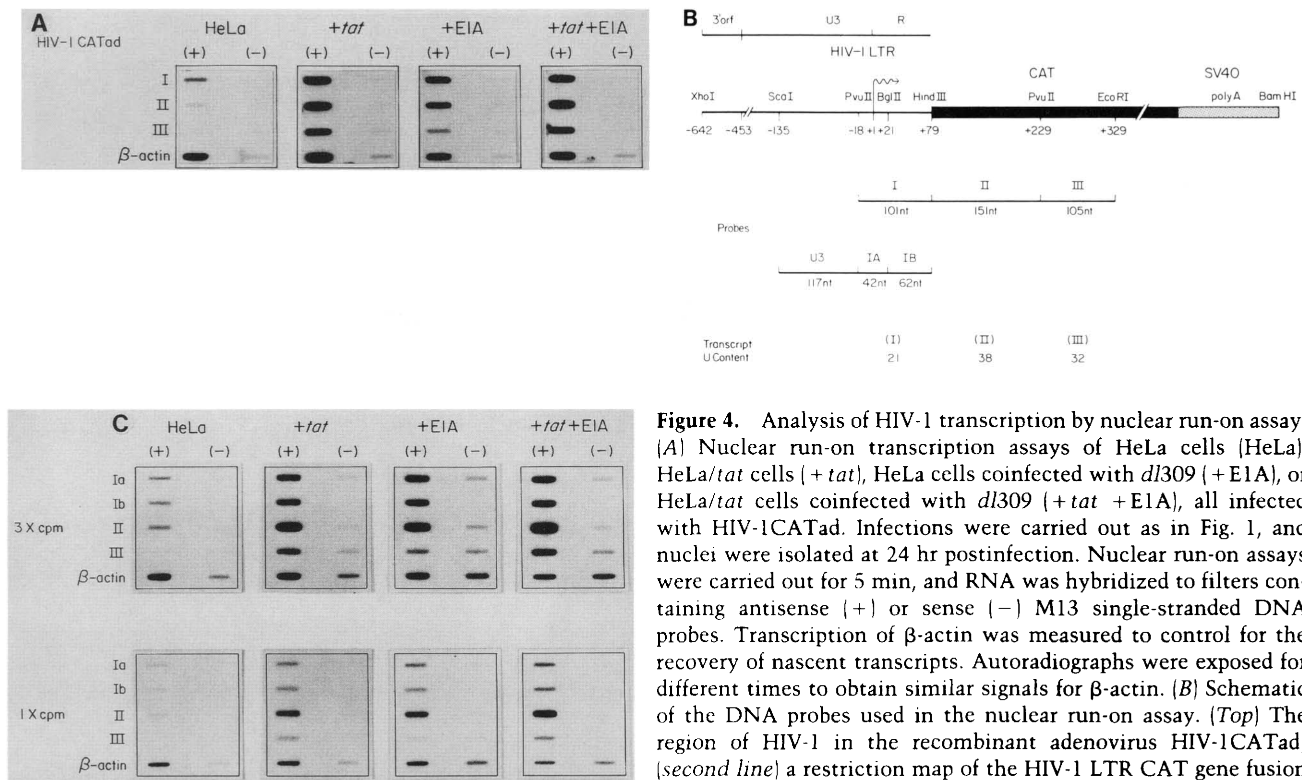

Figure 4. Analysis of HIV-1 transcription by nuclear run-on assay. (*A*) Nuclear run-on transcription assays of HeLa cells (HeLa), HeLa/*tat* cells (+ *tat*), HeLa cells coinfected with *dl*309 (+ E1A), or HeLa/*tat* cells coinfected with *dl*309 (+ *tat* + E1A), all infected with HIV-1CATad. Infections were carried out as in Fig. 1, and nuclei were isolated at 24 hr postinfection. Nuclear run-on assays were carried out for 5 min, and RNA was hybridized to filters containing antisense (+) or sense (−) M13 single-stranded DNA probes. Transcription of β-actin was measured to control for the recovery of nascent transcripts. Autoradiographs were exposed for different times to obtain similar signals for β-actin. (*B*) Schematic of the DNA probes used in the nuclear run-on assay. (*Top*) The region of HIV-1 in the recombinant adenovirus HIV-1CATad; (*second line*) a restriction map of the HIV-1 LTR CAT gene fusion in this virus; (*third* and *fourth lines*) fragments cloned into M13 and used as single-stranded probes for the nuclear run-on assay. Fragment U3 extends from − 135 to − 19; fragment I extends from − 18 to + 83; fragment IA from − 18 to + 24; fragment IB from + 21 to + 83; fragment II from + 78 to + 228; fragment III from + 229 to + 333. (*Bottom*) The uridine contents of the RNA fragments corresponding to probes I, II, and III. (*C*) Nuclear run-on transcription assays of HeLa cells (HeLa), HeLa/*tat* cells (+ *tat*), HeLa cells infected with *dl*309 (+ E1A), or HeLa/*tat* cells coinfected with *dl*309 (+ *tat* + E1A) that were infected with HIV-1CATad. Infections and nuclear run-on assays were performed as in Fig. 4A except that hybridizations and washes were performed at 59°C rather than 65°C. The sense (−) control for fragments IA and IB was fragment I. The higher background hybridization to sense (−) β-actin was due to the lower hybridization temperature.

scriptional polarity in fragments II and III just as it does in the absence of E1A. Therefore, when the level of transcriptional initiation is elevated (by E1A in this case), the primary effect of Tat is to increase elongation: This may afford an explanation for the lack of an effect of Tat on initiation in some systems (Kao et al. 1987). In summary, the data of Figure 4 can account for the stimulation in full-length cytoplasmic transcripts by Tat + E1A compared to E1A alone (Fig. 2A), but the stimulation by Tat + E1A relative to Tat alone still remained to be explained.

Relative contributions of initiation and elongation to trans-*activation*

The observation that transcriptional synergy could not be explained by increased transcription initiation or by a detectable suppression of polarity in the promoter proximal region was puzzling. A possible explanation for this lay in the ability of Tat to stabilize transcribing RNA polymerases, and we wondered whether synergy might become evident with greater distance from the promoter. To address this possibility, we generated additional single-stranded DNA probes corresponding to the promoter distal region by cloning the remainder of the HIV-1-promoted transcription unit into M13 bacteriophages. Fragment IV (+328 to +633) and fragment V (+630 to +866) contain the 3′ part of the CAT gene, whereas fragment VI (+867 to +1476) and fragment VII (+1477 to +1714) contain the SV40 small t intron and polyadenylation signal, respectively (Fig. 5B). These additional probes allowed us to measure the relative transcription rates across the entire HIV-1 LTR-directed transcription unit and permitted estimation of the contributions of initiation and elongation to *trans*-activation. The relative increase in transcription rate in the 3′ end of the transcription unit is a measure of the stimulation of overall transcription of the gene and reflects the maximum potential contribution of transcription to increased gene expression. Transcription rates in the 3′ end of the gene were approximated in fragment VI because the signal of fragment VII in HeLa cells was too low to be quantitated. The relative contribution of elongation was calculated by dividing the increase in overall transcription (fragment VI) by the increase in initiation (fragment I).

Figure 5A shows an autoradiogram of a nuclear run-on assay using contiguous probes that extend across the en-

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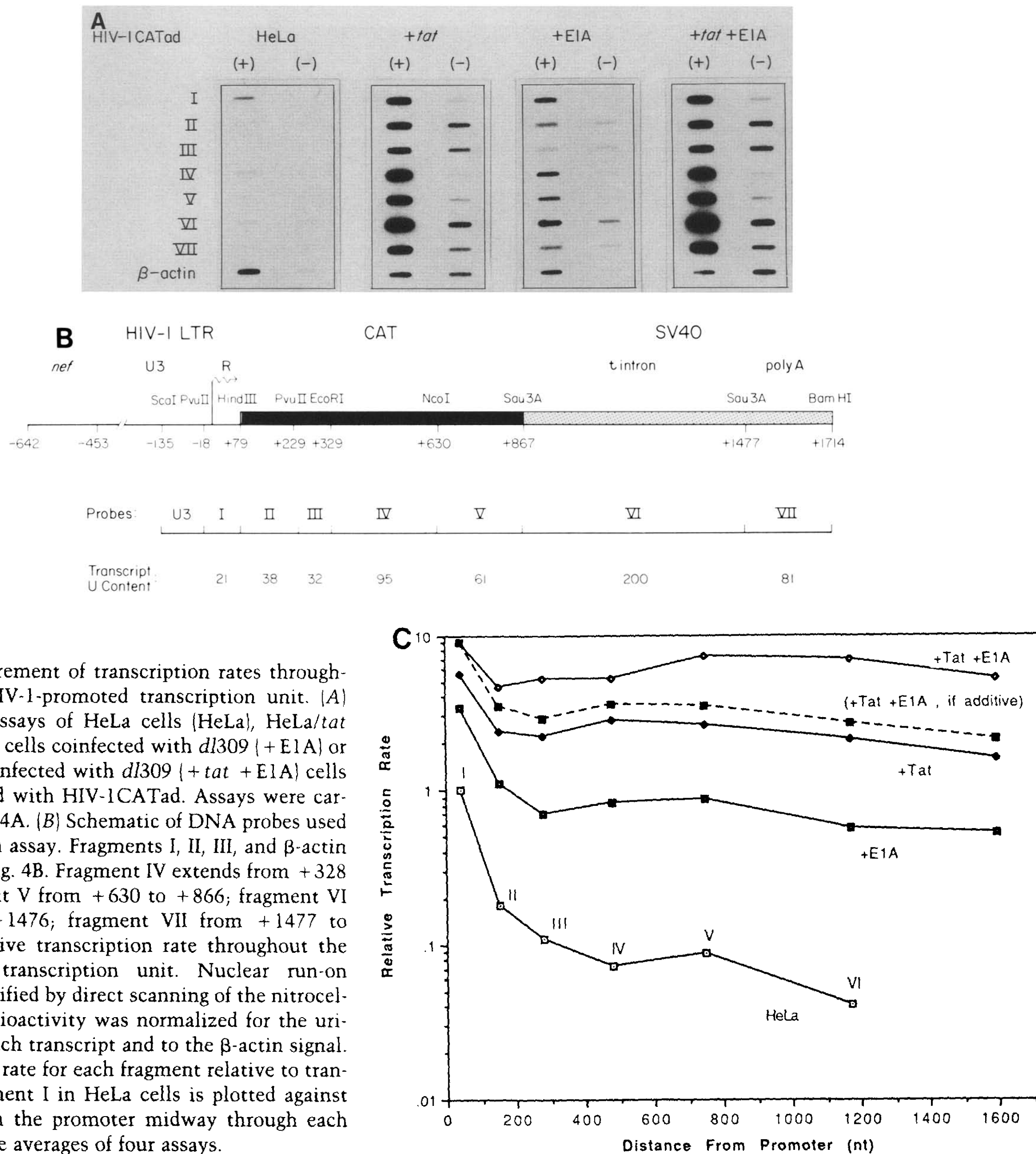

Figure 5. Measurement of transcription rates throughout the entire HIV-1-promoted transcription unit. (*A*) Nuclear run-on assays of HeLa cells (HeLa), HeLa/*tat* cells (+*tat*), HeLa cells coinfected with *dl*309 (+E1A) or HeLa/*tat* cells coinfected with *dl*309 (+*tat* +E1A) cells that were infected with HIV-1CATad. Assays were carried out as in Fig. 4A. (*B*) Schematic of DNA probes used in the nuclear run assay. Fragments I, II, III, and β-actin are described in Fig. 4B. Fragment IV extends from +328 to +633; fragment V from +630 to +866; fragment VI from +867 to +1476; fragment VII from +1477 to +1717. (*C*) Relative transcription rate throughout the HIV-1-promoted transcription unit. Nuclear run-on assays were quantified by direct scanning of the nitrocellulose filters. Radioactivity was normalized for the uridine content of each transcript and to the β-actin signal. The transcription rate for each fragment relative to transcription in fragment I in HeLa cells is plotted against the distance from the promoter midway through each fragment. Data are averages of four assays.

tire HIV-1-directed transcription unit. Table 1 displays quantitation of the relative transcription rate in each fragment for this experiment, after standardization to the β-actin radioactive signal and normalization for the uridine content of each RNA fragment. The relative transcription rates from several experiments are averaged and plotted in Figure 5C.

In the absence of *trans*-activator (Fig. 5A, HeLa), polarity reduced transcription in the 3′ region (fragment VI) to <5% of that in the promoter proximal region (fragment I, Table 1). The distribution of polymerases traversing the entire transcription unit revealed an initial sharp decline in polymerase density in the first 300 nucleotides, followed by a continued decline at a lesser rate (Fig. 5C).

Tat increased promoter proximal transcription ninefold (fragment I) (Fig. 5A; Table 1). In addition, Tat suppressed polarity such that 40% of the initiating RNA polymerases transcribed into fragment VI. With Tat, the density of transcribing RNA polymerases initially declined in the 5′ end of the gene (fragments II and III), although less sharply than in the absence of *trans*-activators; polymerase density remained level in the 3′ part of the gene, unlike in the absence of *trans*-activators. The net result was a larger increase in transcription rates in the promoter distal fragment VI than could be detected in fragments I, II, and III. The increase in overall transcription rates in fragment VI by Tat was 80-fold; therefore, Tat stimulated transcription rates 9-fold due to its ability to stabilize elongation and 9-fold due to its ability to stimulate initiation.

E1A produced a fourfold stimulation in promoter proximal transcription (fragment I) and a smaller, but reproducible, stimulation of elongation, such that 13% of

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Table 1. *Relative transcription rates in the HIV-1 LTR-directed CAT transcription unit*

Fragment	Relative transcription[a–c]			
	HeLa	+Tat	+E1A	+Tat + E1A
I	1.0	9.3 (1.0)	4.0 (1.0)	15.0 (1.0)
II	0.19	3.9 (0.42)	1.0 (0.25)	7.9 (0.53)
III	0.11	3.5 (0.38)	0.68 (0.17)	6.4 (0.43)
IV	0.09	5.7 (0.62)	0.82 (0.20)	9.3 (0.62)
V	0.10	4.6 (0.50)	0.93 (0.23)	12.8 (0.85)
V	0.046	3.7 (0.40)	0.53 (0.13)	11.4 (0.76)
VII	ND[d]	2.5 (0.27)	0.54 (0.13)	8.2 (0.55)

[a]Quantitation of nuclear run-on assay blots, normalized for transcript uridine content and to β-actin transcription rate (to correct for RNA recovery).
[b]Transcription is expressed relative to the transcription in fragment I in HeLa cells.
[c]Numbers in parentheses refer to transcription in the fragment relative to transcription in fragment I in that column.
[d]Signal too low to be accurately quantified.

the initiating RNA polymerases transcribed into fragment VI (compared with 5% in the absence of *trans*-activators). As with Tat, after an initial decrease in fragments II and III, polymerase density stabilized in distal regions of the gene. This led to an E1A effect on elongation that was small in the promoter proximal fragments (fragment II and III) but detectable in the 3′ end of the gene. These results suggest that in addition to increasing initiation, E1A is also able to exert a small stabilizing influence on transcriptional elongation.

Transcriptional synergy is due to increased promoter distal transcription rates

Although the stimulation is difficult to see in the promoter proximal region (fragments I, II, and III), Figure 5A clearly shows that Tat + E1A elicited a greater increase in the transcription of the distal fragments VI and VII than did Tat alone. Quantitation revealed that Tat + E1A produced a 15-fold increase in promoter proximal transcription, which is approximately equal to the sum of their individual effects (Table 1). In addition, Tat + E1A dramatically suppressed polarity, leading to an increase in HIV-1-promoted overall transcription of 250-fold (fragment VI). This stimulation in overall transcription was much larger than could be explained if these two *trans*-activators acted additively (Fig. 5C, dashed line) and is due to suppression of transcriptional polarity because 76% of the initiating RNA polymerases transcribe to the end of the gene in the presence of Tat + E1A, whereas only 40% do so with Tat alone. Thus, Tat + E1A produce an additive stimulation in transcription initiation, but increased elongation accounts for transcriptional synergy.

Synergy between Tat and a phorbol ester

These findings led us to consider the interaction between Tat and other transcriptional activators that influence HIV-1 gene expression. Phorbol 12-myristate 13-acetate (PMA) is capable of stimulating transcription through activation of transcription factors such as AP-1 and NF-κB that interact with common upstream promoter elements (Sen and Baltimore 1986; Angel et al. 1987). Tat and PMA have been shown to stimulate HIV-1 LTR-directed CAT expression synergistically (Nabel and Baltimore 1987; Siekevitz et al. 1987; Tong-Starksen et al. 1987).

Analysis of the effect of Tat and PMA on LTR-directed cytoplasmic RNA levels is shown in Figure 6. Tat produced a 13-fold stimulation in full-length RNA (Fig. 6A, lane 2; Fig. 6B). Like E1A, PMA increased the level of both the full-length and the short transcripts suggesting that it stimulates transcriptional initiation. However, the 2.3-fold stimulatory effect of PMA on the level of the full-length transcripts (Fig. 6A, lane 3; Fig. 6B) was smaller than the 4-fold effect produced by E1A, while the short transcripts appeared to be increased to a greater extent (Fig. 2C). Tat + PMA produced a 141-fold stimulation in the levels of full-length RNA (Fig. 6A, lane 4; Fig. 6B), which was greater than if they acted additively. Similar results were obtained when the cells were treated with PMA for 4 hr (as shown) or 16 hr (data not shown). Thus, PMA cooperates with Tat to elevate HIV-1-promoted RNA levels synergistically. As with E1A, the level of the short transcripts with Tat + PMA was the same as with PMA alone but higher than with Tat alone. These findings indicate that synergy between Tat and PMA may operate through a mechanism similar to that between Tat and E1A.

Discussion

Transcription of HIV-1 genes is regulated by at least two types of *trans*-activators. One class of activator interacts with upstream *cis*-regulatory DNA elements to increase transcriptional initiation. These activators include NF-κB, which interacts with the core enhancer (Nabel and Baltimore 1987); Sp1, which has binding sites upstream of the HIV-1 promoter (Jones et al. 1986); and the adenovirus E1A protein, which interacts with the TATA element (Jones et al. 1988; Nabel et al. 1988; Rice and Mathews 1988b). The second class of activator consists of the HIV-1 Tat protein and its homologs from HIV-2 and SIV, which bind to a *cis*-regulatory RNA element, TAR, in nascent transcripts. Tat can act both to increase

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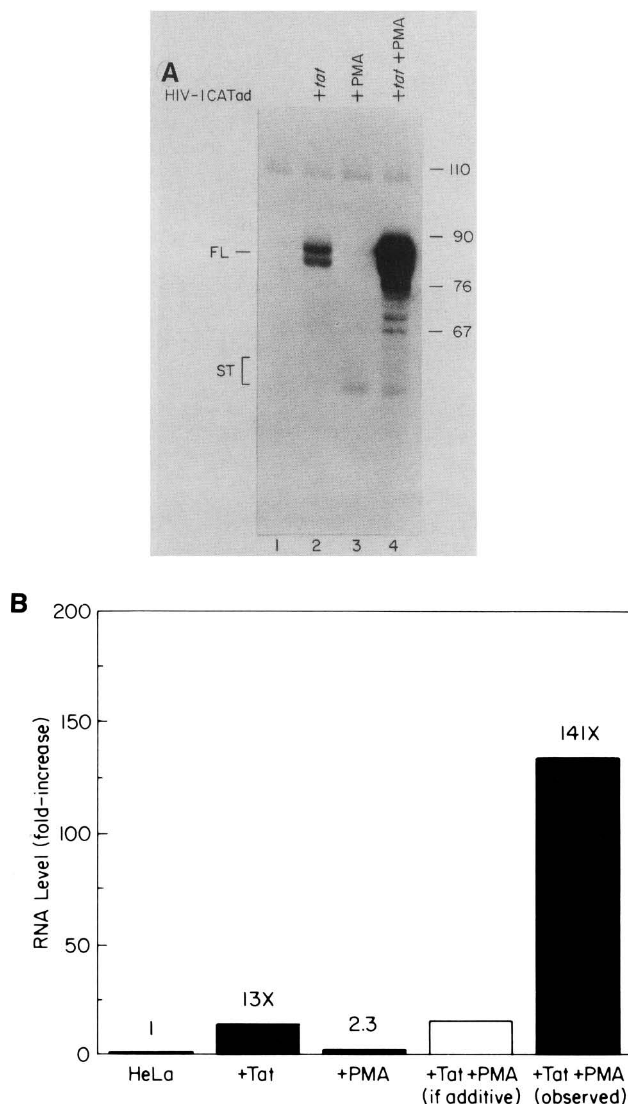

Figure 6. Synergy between Tat and PMA. (*A*) RNase protection assay of cytoplasmic RNA isolated from HIV-1CATad-infected HeLa cells (lane *1*), HeLa/*tat* cells (lane *2*), and HeLa cells or HeLa/*tat* cells treated with 200 ng/ml PMA (lanes *3* and *4*). Markers are *Hpa*II fragments of pBR322 labeled with ^{32}P. HIV-1CATad infections were carried out at an moi of 100, and PMA treatment was at 20 hr postinfection. Cytoplasmic RNA was isolated at 24 hr postinfection and analyzed as in Fig. 2A. Similar results were obtained when PMA was added 8 hr postinfection and RNA was isolated 24 hr postinfection (data not shown). (*B*) Relative HIV-1-directed RNA levels. The stimulation in full-length transcripts relative to HeLa cells was quantified as in Fig. 2C.

transcriptional initiation and stabilize elongation (Laspia et al. 1989). Cooperation between the two classes of *trans*-activator produces a synergistic stimulation of HIV-1 LTR-directed gene expression (Nabel and Baltimore 1987; Tong-Starksen et al. 1987; Rice and Mathews 1988b).

To discover the molecular basis for this synergy, we examined the combined effects of Tat and E1A on HIV-1-directed RNA levels and transcription rates. Synergy between Tat and E1A was accounted for by a stimulation of RNA levels that resulted from an increased rate of LTR-directed transcription. With other cellular promoters, heterologous acidic activators have been shown to stimulate transcription synergistically, possibly by cooperatively increasing the binding of an essential target molecule (Lin et al. 1990). Surprisingly, synergy between Tat and E1A, elucidated by an analysis of RNA polymerase density across the HIV-1-promoted transcription unit, was not caused by increased transcriptional initiation: The combined effect of Tat and E1A on initiation was only additive. Rather, synergy resulted from stimulation of transcriptional elongation. This novel form of cooperation may not be unusual in view of the finding that Tat and the general transcriptional activator PMA exhibited a pattern of regulation of cytoplasmic RNA levels similar to that of Tat + E1A.

Clearly the effect of Tat on initiation is variable in magnitude. In the presence of E1A, Tat stimulated initiation to a lesser extent than it stimulated the basal level of initiation from the HIV-1 promoter. The reduced sensitivity is attributable to saturation of the initiation machinery, perhaps because of limiting transcription factor concentrations or steric hindrance at the promoter. Similarly, it is likely that elevated basal HIV-1 transcription in the transfected COS cell system may explain why Kao et al. (1987) observed a stimulatory effect on elongation but not initiation (M. Kessler and M.B. Mathews, in prep.). This observation could have implications for the development of systems that seek to analyze the stimulation of transcription by Tat in vitro because the magnitude of the initiation effect may be strongly influenced by the strength of the promoter. As shown recently, Tat stimulates transcription in vitro with the primary effect being at the level of elongation (Marciniak et al. 1990).

How is synergy between Tat and E1A achieved at the molecular level? One possible explanation proposes that Tat stabilizes the elongation of the additional initiation complexes (those whose presence on the template is due to E1A) in the same way and to the same extent as it stabilizes elongation of complexes formed in the absence of E1A. However, this explanation is insufficient to account for the degree of suppression of polarity seen, because 75% of initiation complexes reach the 3′ end of the gene with Tat + E1A versus 40% with Tat alone and only 13% with E1A alone. It seems that the cooperative effect on stabilization of elongation may stem from the formation of transcriptional complexes in the presence of both *trans*-activators that are capable of more efficient elongation than those formed with either *trans*-activator alone (Fig. 7). A third possibility, suggested by the finding that E1A also appears to suppress polarity to some extent, is that increasing RNA polymerase density on the template stabilizes elongation, perhaps by altering chromosome structure or conformation.

It is clear from these and other studies (Kao et al. 1987; Laspia et al. 1989; Ratnasabapathy et al. 1990) that Tat makes a major contribution to transcriptional elon-

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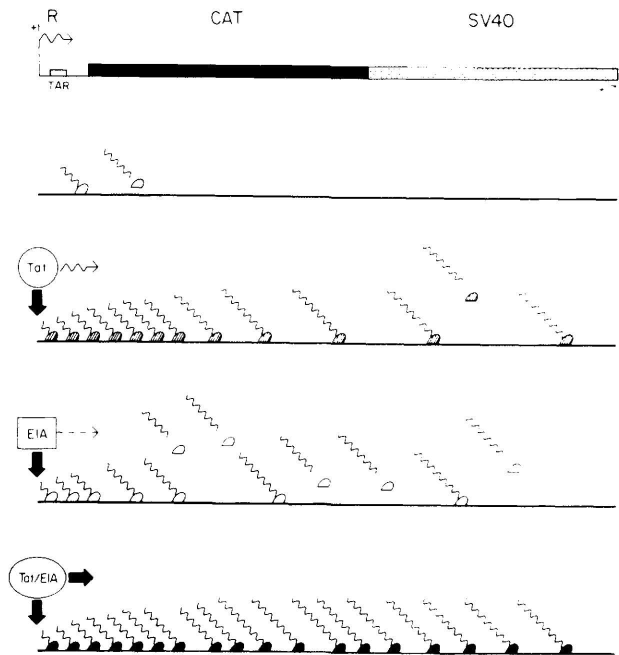

Figure 7. Model for synergy between Tat and E1A. In the absence of *trans*-activators, transcriptional initiation is low and elongation is unstable. Tat increases transcriptional initiation and stabilizes elongation. E1A increases initiation and has a small stabilizing effect on elongation. Tat and E1A cooperate to promote the formation of a highly processive RNA polymerase complex resulting in a synergistic increase in polymerase density in the 3′ end of the transcription unit with only an additive effect on initiation.

gation. In some of the experiments shown here (Fig. 5), increased elongation appears to accompany increased initiation, suggesting that elongation may be aided by increasing rates of initiation. However, the effect of Tat on elongation is a specific one because under conditions where Tat and E1A produce similar increases in initiation Tat always suppresses polarity to a greater extent than E1A (Fig. 4A,C; Laspia et al. 1989). Peterlin and colleagues proposed that Tat acts as an antiterminator, permitting readthrough of transcription complexes blocked in the TAR region (Kao et al. 1987; Selby et al. 1989). We find that most transcription termination occurs in the first 400 nucleotides of the HIV-1-directed transcription unit. Rather than acting to relieve a block to transcription at a specific terminator site, as occurs at *nut* sites in the leftward promoter of bacteriophage λ (Roberts 1988), we favor the idea that Tat stimulates elongation through a number of critical regions in the HIV-1 leader and the CAT sequences where pausing or termination occurs (M. Kessler and M.B. Mathews, in prep.). Thus, the short RNAs whose 3′ ends map to the base of the stem–loop formed by the HIV-1 leader RNA may be formed by the processing of longer, but incomplete, transcripts produced by termination or pausing downstream. This idea is supported by analysis of the short HIV RNAs generated in vitro (Toohey and Jones 1989).

Elongational instability is likely to be specified at the promoter through an interaction at the DNA level because mutations that abolish the structure of TAR RNA do not relieve polarity (Laspia et al. 1989). It appears that delivery of Tat to the HIV-1 promoter is sufficient to increase LTR-directed transcription rates (Southgate et al. 1990). Tat may exert its influence on HIV-1 transcription by contacting RNA polymerase or transcription factors such as TFIID directly or, alternatively, by acting through upstream activators such as Sp1 and NF-κB. In support of this theory, Sp1 deletion mutants show reduced stimulation of HIV-1 RNA levels by Tat but not by E1A (M. Laspia, unpubl.). As a unifying hypothesis, we propose that Tat mediates the formation of an initiation complex containing certain constellations of upstream factors and facilitates the stable binding of elongation factors. Alternatively, Tat may act as an elongation factor itself or work through other elongation factor(s) that interact with the postinitiation RNA polymerase and thereby promote stable elongation.

Synergy between Tat and general transcriptional activators has potential implications for the regulation of HIV-1 gene expression. Early in infection or during viral latency, the HIV-1 LTR is relatively inactive, possibly because levels of Tat protein are rate limiting. Stimulation by antigen or cytokines induces the LTR to become transcriptionally active due to the induction or activation of cellular transcription factors (Cullen and Greene 1989; Pomerantz et al. 1990). Because of transcriptional polarity, most of the RNA polymerases that initiate transcription terminate prematurely. However, once Tat has accumulated, it may act in concert with activated transcription factors in the cell to cause a large increase in gene expression. This burst of transcriptional activity may serve as a molecular trigger that induces HIV-1 to transit from latency to active viral growth.

During our analysis of the basis for synergy, we noticed a discrepancy between the relative increase in reporter enzyme activity and reporter RNA levels. Similar discrepancies have been reported previously and ascribed to post-transcriptional regulation by Tat (Cullen 1986; Feinberg et al. 1986; Rosen et al. 1986; Wright et al. 1986). Recent studies have provided compelling evidence for a translational regulation by Tat in *Xenopus* oocytes (Braddock et al. 1989, 1990). Other workers, however, have failed to observe a post-transcriptional effect (Peterlin et al. 1986; Jeang et al. 1988; Rice and Mathews 1988a). In the experiments described here, we found that both E1A and Tat increased the utilization of TAR–CAT RNA and that a correlation exists between the level of cytoplasmic CAT RNA and the efficiency with which it was utilized to produce CAT enzyme. It is therefore possible that the apparent post-transcriptional effect of Tat is seen only in systems possessing low basal levels of HIV-1 expression. Moreover, the finding that two different *trans*-activators increased TAR–CAT RNA utilization suggests that the effect is a function of increased mRNA level and not a specific action of Tat. The alternative, that E1A and Tat might both act specifically to increase translation of the TAR–CAT RNA, is, in our view, much less likely.

In conclusion, our findings provide an explanation for

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the synergistic stimulation of HIV-1 gene expression produced by Tat and general transcriptional activators. Furthermore, they underscore the importance of the ability of Tat to stabilize transcriptional elongation, in conjunction with its ability to stimulate initiation, in the regulation of HIV-1 transcription. In addition, we provide a possible explanation for the variable magnitude of the Tat/TAR effect on transcriptional initiation. We propose that Tat and general transcription activators can cooperate to cause the formation at the HIV-1 promoter of an initiation complex capable of very stable transcriptional elongation.

Materials and methods

Adenoviruses, cell cultures, and infections

HIV-1CATad contains HIV-1 sequences −642 to +83 (Rice and Mathews 1988a,b). E1A was supplied by infection with the phenotypically wild-type adenovirus 5 *dl*309 (Jones and Shenk 1979).

HeLa cells and HeLa/*tat* cells were kindly provided by K. Valerie (Smith, Kline and French Laboratories; Valerie et al. 1988). HeLa cells were grown in Dulbecco's modified medium supplemented with 10% fetal bovine serum, and HeLa/*tat* cells were maintained in medium containing 200 μg/ml geneticin.

Approximately 7.5×10^6 cells in 10-cm dishes were infected at 37°C with a recombinant adenovirus in Dulbecco's modified medium supplemented with 2% gamma-globulin-free serum (Laspia et al. 1989). At 2 hr postinfection the medium was replaced with fresh medium containing 10 mM hydroxyurea to prevent recombinant adenovirus replication, especially in the case of coinfection with *dl*309. Cells were washed in phosphate-buffered saline (PBS) and harvested by scraping in PBS.

Plasmid construction

Antisense (+) and sense (−) single-stranded DNA probes used in the nuclear run-on assay were M13 clones containing HIV-1 LTR sequences derived from pU3RIII (Sodroski et al. 1985a). Construction of probes U3, I, IA, 1B, II, III, and β-actin have been described (Laspia et al. 1989). Additional M13 probes constructed for this study are probe IV +328 (*Eco*RI) to +633 (*Nco*I); probe V +639 to +866 (*Sau*3A); probe VI +867 to +1476 (*Sau*3A); and probe VIII +1477 (*Bcl*I) to +1717 (*Bam*HI). Probe VI corresponds to SV40 sequences 4713–4104, and probe VII to sequences 2770–2553.

CAT assays

Cells from one 10-cm plate were harvested and resuspended in 0.25 M Tris (pH 8.0) and lysed by three freeze/thaw cycles. Lysates (50 μl), from one-sixth of a plate, were assayed for CAT activity as described (Gorman et al. 1982). Thin-layer chromatograms were quantified with an Ambis Betascan System (San Diego, CA). CAT activity is presented in terms of units where 1 unit is defined as the conversion of 1% chloramphenicol to the acetylated form in a 1-hr reaction at 37°C. Extracts with high activity were diluted or incubated for shorter times so that <50% of the substrate was acetylated, ensuring that the assay was in the linear range.

RNA isolation and RNase protection assay

Cytoplasmic RNA was prepared following lysis with Nonidet P-40 as described (Anderson et al. 1974). Homologous antisense riboprobe was synthesized from linearized pGEM23 vectors, and the RNase protection assay was performed as described (Laspia et al. 1989). Briefly, 5 μg of RNA and 1×10^6 cpm of riboprobe in 30 μl hybridization buffer were denatured and incubated overnight at 40°C. The mixture was then digested with 10 μg/ml RNase A and 10 μg/ml RNase T1 in 300 μl of digestion buffer at room temperature. RNase digestion products were separated by electrophoresis in 8% polyacrylamide–7 M urea sequencing gels. Controls were performed to show that assay was in the linear range. Protected fragments were visualized by autoradiography with an intensifying screen and quantified by direct scanning of dried gels with a Betagen radioanalytic imaging system.

Nuclear run-on transcription assay

Recombinant adenovirus-infected cells were harvested at 24 hr postinfection, and nuclei were prepared and stored in liquid nitrogen. Isolation of nuclei and nuclear run-on assays were performed as described by Greenberg and Ziff (1984), with minor modifications (Laspia et al. 1989). Briefly, 3×10^7 nuclei were thawed, mixed with an equal volume of twofold concentrated transcription buffer containing 200 μCi [α-^{32}P]UTP (3000 Ci/mM), and incubated for 5 min at 30°C. Reactions were stopped by the addition of 0.6 ml of 10 mM Tris (pH 7.4), 50 mM $MgCl_2$, 2 mM $CaCl_2$, 500 mM NaCl, 40 μg/ml of DNase I, and 3.3 μg/ml α-amanitin. RNA was isolated, fragmented with 0.2 N NaOH for 15 min on ice, and neutralized with HEPES buffer. M13 DNA probes (5 μg) were attached to nitrocellulose filters using a slot blot apparatus. RNA hybridization and RNase treatment of the filters were performed as described (Greenberg and Ziff 1984). Filters were subjected to autoradiography with an intensifying screen. Bound radioactivity was quantified with a Betagen radioanalytic imaging system.

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Synergy between HIV-1 Tat and adenovirus E1A is principally due to stabilization of transcriptional elongation.

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