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 quiescence 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. Similarly, 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; Sadai et al. 1988, Laspia et al. 1989), but evidence for post-transcriptional regulation also exists (Cullen 1986; Fein-
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.](image-url)
HIV transcriptional synergy due to elongation

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-I CATad-infected HeLa cells [lane 1], HeLa/tat [lane 2], HeLa cells coinfectd with dl309 [lane 3], or HeLa/tat coinfectd with dl309 [lane 4]. Markers are *HpaI fragments of pBR322 labeled with 32P. 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-I CATad contains the HIV-I LTR sequence -642 to -1-83. The homologous antisense riboprobe was synthesized from pGEM23 linearized with XhoI. Full-length RNA transcripts initiating at +1 of the HIV-I 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.

Tat produced a slight reduction in the level of short transcripts in Figure 2A, although the short transcripts remained unchanged in other experiments [Lasplia 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
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 + EIA. This discrepancy was not Tat-specific, however, as an increase was also seen with EIA 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 EIA, Tat, or Tat + EIA. 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 Matthews 1988a). Another interpretation, which in our view is less likely, is that Tat and EIA 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; Sengupta and Silverman 1989; Gunny et al. 1990; Sengupta et al. 1990). However, post-translational mechanisms, such as concentration-dependent changes in CAT enzyme stability, also cannot be excluded.

Synergy is not due to increased transcriptional initiation

To pursue further the mechanism underlying the synergy between Tat and EIA 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-32P]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 radioactive hybridization 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 EIA 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 EIA 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 EIA to increase promoter proximal transcription.

As shown earlier, Tat also suppressed the polar effect on transcription in the CAT sequences, whereas EIA did not. Unexpectedly, simultaneous expression of Tat and EIA did not elicit any obvious increase in transcription in fragment I relative to either Tat or EIA alone, although quantitation of subsequent experiments revealed a nearly additive effect [see below]. Furthermore, Tat + EIA 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 + EIA on HIV-1-directed RNA levels.

In the adjacent region of the CAT gene (fragments II and III), Tat + EIA 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 + EIA produced an increase over EIA alone, indicating that when transcriptional initiation is elevated by EIA, Tat suppresses tran-
TRANSCRIPTIONAL SYNERGY DUE TO ELONGATION

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 dl309 (+ E1A), or HeLa/tat cells coinfected with dl309 (+ tat + E1A), all infected with HIV-1CATd. 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-1CATd, [second line] a restriction map of the HIV-1 LTR CAT gene fusion in this virus; [third and fourth lines] fragments cloned into M13 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 transcription. 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-
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 coinfectd with d309 (+ E1A) or HeLa/tat cells coinfectd with d309 (+ 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 +867; 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.

The HIV-1-directed transcription unit. Table 1 displays quantification 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...
Table 1. Relative transcription rates in the HIV-1 LTR-directed CAT transcription unit

<table>
<thead>
<tr>
<th>Fragment</th>
<th>HeLa</th>
<th>+ Tat</th>
<th>+ EIA</th>
<th>+ Tat + EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.0</td>
<td>9.3 (1.0)</td>
<td>4.0 (1.0)</td>
<td>15.0 (1.0)</td>
</tr>
<tr>
<td>II</td>
<td>0.19</td>
<td>3.9 (0.42)</td>
<td>1.0 (0.25)</td>
<td>7.9 (0.53)</td>
</tr>
<tr>
<td>III</td>
<td>0.11</td>
<td>3.5 (0.38)</td>
<td>0.68 (0.17)</td>
<td>6.4 (0.43)</td>
</tr>
<tr>
<td>IV</td>
<td>0.09</td>
<td>5.7 (0.62)</td>
<td>0.82 (0.20)</td>
<td>9.3 (0.62)</td>
</tr>
<tr>
<td>V</td>
<td>0.10</td>
<td>4.6 (0.50)</td>
<td>0.93 (0.23)</td>
<td>12.8 (0.85)</td>
</tr>
<tr>
<td>V</td>
<td>0.046</td>
<td>3.7 (0.40)</td>
<td>0.53 (0.13)</td>
<td>11.4 (0.76)</td>
</tr>
<tr>
<td>VII</td>
<td>ND*</td>
<td>2.5 (0.27)</td>
<td>0.54 (0.13)</td>
<td>8.2 (0.55)</td>
</tr>
</tbody>
</table>

*Quantitation of nuclear run-on assay blots, normalized for transcript uridine content and to β-actin transcription rate (to correct for RNA recovery).

+Transcription is expressed relative to the transcription in fragment I in HeLa cells.

• Numbers in parentheses refer to transcription in the fragment relative to transcription in fragment I in that column.

• 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 EIA 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, EIA 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 + EIA elicited a greater increase in the transcription of the distal fragments VI and VII than did Tat alone. Quantitation revealed that Tat + EIA 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 + EIA 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 + EIA, whereas only 40% do so with Tat alone. Thus, Tat + EIA 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 EIA, 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 EIA, 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 EIA, 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 EIA.

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 EIA 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...
Synergy between Tat and PMA 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.

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 HpaI fragments of pBR322 labeled with 32P. 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 post-infection 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.
In some of the experiments shown here (Fig. 5), we favor the idea that Tat stimulates elongation through unit. Rather than acting to relieve a block to transcription factors such as TFII-D directly or, alternatively, by acting through upstream activators such as Sp1 and NF-kB. 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
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 d309 (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 × 10⁶ 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 d309. 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 pUXIII (Sodroski et al. 1985a). Construction of probes U3, I, 1A, 1B, II, III, and β-actin have been described (Laspia et al. 1989). Additional M13 probes constructed for this study are probe IV +328 [EcoRI] to +633 [NcoI]; probe V +639 to +866 [SacII]; probe VI +867 to +1476 [SacII]; and probe VIII +1477 [BglII] to +1717 [ BamHI]. 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 (see below). Extracts 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 radiolytic 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 × 10⁶ nuclei were thawed, mixed with an equal volume of twofold concentrated transcription buffer containing 200 μCi [α-³²P]UTP (3000 Ci/mmol), and incubated for 5 min at 30°C. Reaction were stopped by the addition of 0.6 ml of 10 mM Tris (pH 7.4), 50 mM MgCl₂, 2 mM CaCl₂, 500 mM NaCl, 40 μg/ml of DNase I, and 3.3 μg/ml a-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 radiolytic 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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