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Transfer of Tat and release of TAR RNA during the activation of the human immunodeficiency virus type-1 transcription elongation complex

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The HIV-1 *trans*-activator protein, Tat, is a potent activator of transcriptional elongation. Tat is recruited to the elongating RNA polymerase during its transit through the *trans*-activation response region (TAR) because of its ability to bind directly to TAR RNA expressed on the nascent RNA chain. We have shown that transcription complexes that have acquired Tat produce 3-fold more full-length transcripts than complexes not exposed to Tat. Western blotting experiments demonstrated that Tat is tightly associated with the paused polymerases. To determine whether TAR RNA also becomes attached to the transcription complex, DNA oligonucleotides were annealed to the nascent chains on the arrested complexes and the RNA was cleaved by RNase H. After cleavage, the 5′ end of the nascent chain, carrying TAR RNA, is quantitatively removed, but the 3′ end of the transcript remains associated with the transcription complex. Even after the removal of TAR RNA, transcription complexes that have been activated by Tat show enhanced processivity. We conclude that Tat, together with cellular co-factors, becomes attached to the transcription complex and stimulates processivity, whereas TAR RNA does not play a direct role in the activation of elongation and is used simply to recruit Tat and cellular co-factors.
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Introduction

Gene expression can be regulated either by varying the rate of transcription initiation or by controlling transcriptional elongation by RNA polymerase II. Although contemporary research has focused primarily on the mechanisms used to regulate initiation, it is known that the expression of several eukaryotic genes, including the c-*myc*, c-*fos* and heat shock genes, is controlled principally by the clearance of blocks to transcription elongation downstream of the promoter (Wright and Bishop, 1989; Ramamurthy *et al.*, 1990; Spencer and Groudine, 1990; Spencer *et al.*, 1990; Mechti *et al.*, 1991; Rasmussen and Lis, 1993; Krumm and Groudine, 1995; Krumm *et al.*, 1995; Li *et al.*, 1996). The two processes may be linked. Recent evidence suggests that enhancer elements stimulate transcription initiation by polymerases with increased processivity (Yankulov *et al.*, 1994; Krumm *et al.*, 1995; Blair *et al.*, 1996).

The biochemical mechanisms used to regulate RNA polymerase processivity are poorly understood. A variety of cellular factors including TFIIS (Reines *et al.*, 1989; Christie *et al.*, 1994; Cipres-Palacin and Kane, 1994), TFIIF (Tan *et al.*, 1995) and elongin (Aso *et al.*, 1995; Kibel *et al.*, 1995; Pause *et al.*, 1996) can modify RNA polymerase II elongation rates. Transcription elongation and promoter clearance are also regulated by phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II by TFIIH (Laybourn and Dahmus, 1990; O'Brien *et al.*, 1994; Yankulov *et al.*, 1995), and several other kinases including the P-TEFb elongation factor (Marshall *et al.*, 1996). However, each of these factors appears to be part of the basic transcription machinery, and the biochemical basis for gene-specific control of elongation has not been discovered.

The human immunodeficiency virus (HIV) *trans*-activator protein (Tat) is the first example of a viral regulatory protein that controls elongation by RNA polymerase II (Kao *et al.*, 1987; Laspia *et al.*, 1989; Ratnasabapathy *et al.*, 1990). In the absence of Tat, the majority of RNA polymerases initiating transcription stall near the promoter. Following the addition of Tat, there is a dramatic increase in the density of RNA polymerases found downstream of the promoter (Kao *et al.*, 1987; Laspia *et al.*, 1989, 1990; Feinberg *et al.*, 1991; Marciniak and Sharp, 1991; Kato *et al.*, 1992; Rittner *et al.*, 1995).

Tat activity requires the *trans*-activator response element (TAR), an RNA element located at the 5′ end of the nascent transcript which forms a stem–loop structure of 59 nucleotides (Rosen *et al.*, 1985; Cullen, 1986; Muesing *et al.*, 1987; Berkhout *et al.*, 1989; Selby *et al.*, 1989), and acts as a binding site for Tat (Dingwall *et al.*, 1989). Tat recognizes a tri-nucleotide bulge and flanking base pairs located near the apex of the TAR RNA stem–loop structure (Dingwall *et al.*, 1990; Roy *et al.*, 1990a; Weeks *et al.*, 1990; Calnan *et al.*, 1991; Harper and Logsdon, 1991; Weeks and Crothers, 1991; Delling *et al.*, 1992; Churcher *et al.*, 1993; Hamy *et al.*, 1993). Binding of Tat to TAR induces a conformational change in TAR that repositions critical functional groups on the RNA for high affinity interactions with the protein (Puglisi *et al.*, 1992; Aboul-ela *et al.*, 1995, 1996; Brodsky and Williamson, 1997). Sequences in the apical loop sequence of TAR RNA are also essential for Tat activation of transcription, although this region is not required for Tat binding (Feng and Holland, 1988; Berkhout and Jeang, 1989; Selby *et al.*, 1989; Dingwall *et al.*, 1990; Roy *et al.*, 1990b; Churcher *et al.*, 1993). It seems likely that cellular co-factors for Tat are recruited through interactions with the

TAR RNA loop sequence (Sheline *et al.*, 1991; Wu *et al.*, 1991; Zhou and Sharp, 1995, 1996).

The development of efficient cell-free transcription systems that respond to Tat has greatly advanced the analysis of the *trans*-activation mechanism (Marciniak *et al.*, 1990; Marciniak and Sharp, 1991; Kato *et al.*, 1992; Graeble *et al.*, 1993; Laspia *et al.*, 1993; Churcher *et al.*, 1995; Parada *et al.*, 1995; Rittner *et al.*, 1995; Zhou and Sharp, 1995, 1996; Keen *et al.*, 1996; Parada and Roeder, 1996). These studies have shown that the activation of transcription by Tat takes place at a distinct step following the initiation of transcription. In the cell-free systems, the total level of initiation remains constant in the presence or absence of Tat, but addition of Tat produces a dramatic increase in the amount of longer transcripts (Marciniak and Sharp, 1991; Kato *et al.*, 1992; Graeble *et al.*, 1993; Laspia *et al.*, 1993; Rittner *et al.*, 1995). Further evidence that Tat activation of RNA polymerase processivity is a post-initiation event stems from the observation that TAR RNA elements are functional even when they are placed several hundred nucleotides downstream of the start of transcription (Churcher *et al.*, 1995; Wright and Luccarini, 1996).

There do not appear to be specific elements in the HIV promoter that direct transcription by a specialized Tat-responsive polymerase (Kato *et al.*, 1992; Laspia *et al.*, 1993; Rittner *et al.*, 1995). Mutations in each of the critical promoter elements of the HIV long terminal repeat (LTR) that are required for initiation in the cell-free system (the Sp1 sites, TATA box and INR elements) reduce the efficiency of transcription initiation, but do not limit the ability of the polymerases that have initiated transcription to respond to Tat (Rittner *et al.*, 1995). Efficient Tat responses are also observed when TAR is placed downstream of heterologous promoters, such as the adenovirus major late promoter or the c-*myc* gene promoter (Laspia *et al.*, 1993; Wright *et al.*, 1994; Rittner *et al.*, 1995).

These experiments suggest that Tat acts analogously to the bacteriophage λN anti-terminator protein (Barik *et al.*, 1987; Whalen and Das, 1990; Nodwell and Greenblatt, 1991; Greenblatt *et al.*, 1993; Mogridge *et al.*, 1995) and forms a complex with RNA polymerase during its transit through TAR RNA (Kao *et al.*, 1987; Dingwall *et al.*, 1990; Feinberg *et al.*, 1991; Churcher *et al.*, 1995; Rittner *et al.*, 1995). This reaction probably involves not only the binding of Tat to TAR RNA found on the 5′ end of the nascent chain, but also the recruitment of cellular co-factors, including the TAR RNA loop-binding proteins and specialized cellular elongation factors (Wu *et al.*, 1991; Herrmann and Rice, 1995; Wu-Baer *et al.*, 1995a; Zhou and Sharp, 1995, 1996). In support of this hypothesis, we recently have demonstrated that Tat forms an integral component of the activated transcription elongation complex (Keen *et al.*, 1996).

When the λN protein interacts with its RNA recognition element, a stem–loop structure called the *nut* site, it forms a stable complex with the nascent RNA chain, the elongating RNA polymerase and several cellular co-factors (Mogridge *et al.*, 1995). Here we test whether TAR RNA also becomes stably attached to the elongating RNA polymerase. The results show, surprisingly, that although TAR is used to recruit Tat protein to the polymerase, this reaction is transient, and the continued presence of TAR is not required after the activation of the transcription complex.

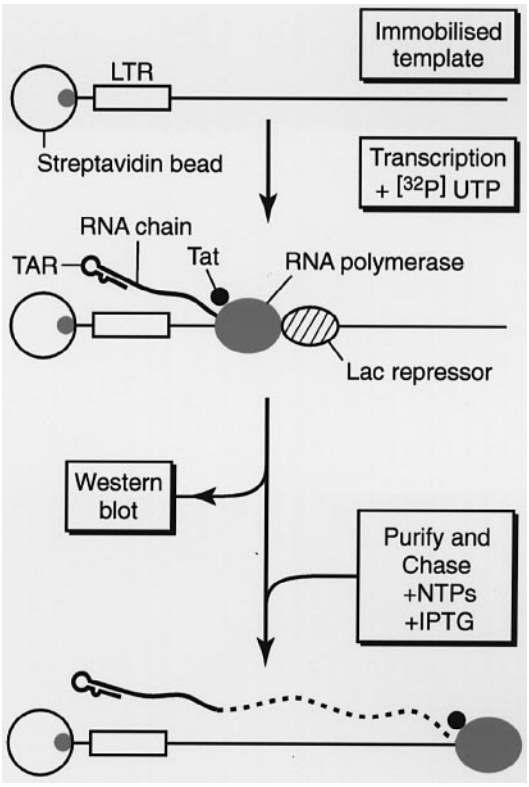


Fig. 1. Strategy for analysing the behaviour of purified transcription complexes. Top: cell-free transcription reactions were performed using biotinylated template DNA immobilized on streptavidin-coated beads. To trap the elongating polymerase, *lac* repressor protein (LacR) is bound to the *lac* operator site on the template. Nascent RNA chains are labelled by incorporation of [α-^{32}P]UTP. Middle: transcription complexes arrested at LacR are purified by isolating the streptavidin-coated beads and repeated washing. Bottom: the protein composition of the purified transcription complexes can be analysed by Western blots using antibodies to RNA polymerase and Tat. Alternatively, the ability of the transcription complexes to elongate was measured by 'chasing' with unlabelled NTPs after dissociation of LacR from the templates following the addition of IPTG.

Results

Purification of transcription complexes arrested by LacR

In a previous study, we developed a method to 'trap' and purify actively elongating RNA polymerases using the *lac* repressor (LacR) as a block to elongation (Keen *et al.*, 1996). The principle of this method is outlined in Figure 1. Biotinylated DNA templates carrying the *lac* operator cloned downstream of the HIV LTR were used in cell-free transcription reactions performed in the presence of LacR. In contrast to terminator sequences carrying RNA stem–loop structures, which induce dissociation from the template, the elongating transcription complexes that have been arrested by LacR remain stably bound to the DNA (Deuschle *et al.*, 1990; Kuhn *et al.*, 1990; Reines and Mote, 1993). This permits the purification of the arrested transcription complexes following attachment of the biotinylated template to streptavidin-coated beads. Polymerases that have been trapped by LacR are functional and can resume transcription after the addition of unlabelled

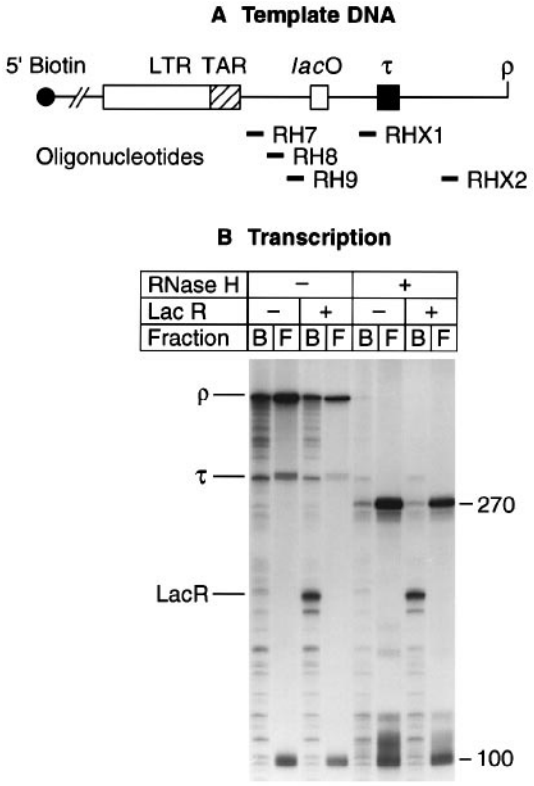


Fig. 2. Purification of arrested transcription complexes. (**A**) Structure of the template DNA. The positions of the LTR, TAR, *lac* operator (*lac*O), synthetic terminator (τ) and transcription run-off at the end of the template (ρ) are indicated. The sequences complementary to the oligonucleotides used to direct RNase H cleavage of the nascent chains are shown below the template DNA structure. Oligonucleotides RHX1 (nucleotides 270–290) and RHX2 (400–420) were used to remove RNA transcripts that had read through LacR. Oligonucleotides RH7 (76–86), RH8 (104–119) and RH9 (151–166) were used to cleave the nascent RNA chain after TAR. (**B**) Purification of transcription complexes arrested at LacR. Transcription reactions were performed using immobilized template DNA carrying a wild-type TAR element in the presence or absence of 1 μg of LacR and 200 ng of recombinant Tat protein. After labelling for 20 min with [α-^{32}P]UTP, the immobilized templates were purified from the reaction mixture and the RNA bound to the template (B) and the free RNA (F) were analysed by PAGE. In the absence of *lac* repressor, the major transcription products are found at the end of the template (ρ) or at the synthetic terminator sequence (τ). Addition of LacR to the reaction (lanes 3, 4, 7 and 8) results in accumulation of a new RNA product due to the arrest of RNA polymerases by LacR. The arrested polymerases remain attached to the template and are found exclusively in the bound fraction. To remove nascent RNA chains on polymerase that have transcribed beyond LacR but remain attached to the template DNA, the nascent RNA was hybridized to the RHX1 and RHX2 oligonucleotides and then digested with RNase H (lanes 5–8). Cleavage of the full-length RNA by these oligonucleotides produces fragments of 100 and 270 nt which are released from the template (lanes 6 and 8). Following cleavage, the major transcript that remains bound to the template corresponds to polymerases arrested at LacR (lane 7).

nucleoside triphosphates (NTPs). These 'chase' experiments can be used to measure the effects of Tat on polymerase elongation.

The templates used in our experiments also carry a synthetic terminator (Graeble *et al.*, 1993) cloned downstream of the *lac* operator (Figure 2A). This terminator sequence (τ) creates a block to transcription elongation that can be overcome by Tat (Graeble *et al.*, 1993; Churcher *et al.*, 1995; Rittner *et al.*, 1995; Keen *et al.*, 1996). Thus, it is possible to detect Tat activation of transcription by measuring the ratio of transcripts that terminate at τ and at the end of the template (ρ).

As shown in Figure 2B, the distribution of RNA polymerases on the purified templates can be deduced from the length of the ^{32}P-labelled nascent RNA chains that co-purify with the template. In the absence of added LacR, the majority of the transcripts that reach the end of the template (ρ) or the terminator sequence (τ) dissociate from the templates and are found in the free fraction. This fraction also contains end-labelled RNA fragments of ~100 nt which are found in the HeLa cell extract. However, ~25% of the transcripts produced in the reaction remain bound to the template. These transcripts are heterogeneous in size, but the most prominent bands correspond to transcription complexes that have become arrested at either ρ or τ.

Addition of LacR creates a new block to the transcription elongation. As shown in Figure 2B, between 50 and 80% of the polymerases engaged in transcription become arrested at this site, giving rise to a new transcript of 190 nt (labelled LacR in the figures). These transcripts are found exclusively in the bound fraction, demonstrating that transcription complexes paused by LacR remain tightly associated with the template (Deuschle *et al.*, 1990; Kuhn *et al.*, 1990; Reines and Mote, 1993; Keen *et al.*, 1996).

Although the majority of the purified transcription complexes pause at LacR, a small number of polymerases that have transcribed through the block can be found attached to the template at more distal sites (primarily at ρ and τ). The presence of labelled transcripts downstream of the repressor limits the sensitivity of 'chase' experiments since any new signal which is generated during a chase would have to be detected above this background. To circumvent this problem, we removed the labelled nascent chains present on transcription complexes paused upstream of LacR using an RNase H digestion procedure. After labelling for 20 min, two DNA oligonucleotides, RHX1 and RHX2 (complementary to nucleotides 270–290 and 400–420, respectively) were added together with RNase H and reactions were continued for a further 5 min. The oligonucleotides anneal to the nascent chains that extend beyond LacR and direct the digestion of the RNA by the RNase H. The cleaved RNAs are then released into the free fraction. As shown in Figure 2B, when RHX1 and RHX2 are present, the cleavage products of the expected size (100 and 270 nt) are found exclusively in the free fraction. As expected, transcripts terminating at the repressor are unaffected by the RNase H digestion procedure, and the bound fraction contains almost exclusively labelled transcripts derived from the complexes arrested at LacR.

In addition to the transcripts that extend beyond LacR, the purified templates also carry a low level of polymerases that have paused prior to reaching LacR and give rise to a number of short transcripts. The amount of these short transcripts can be minimized by adding 250 μM unlabelled UTP during the RNase H digestion step. During this 'pre-chase' (performed in the presence of LacR), the prematurely stalled polymerases either dissociate from the template or resume transcription and reach the pause site at LacR. After the combined RNase H treatment and the

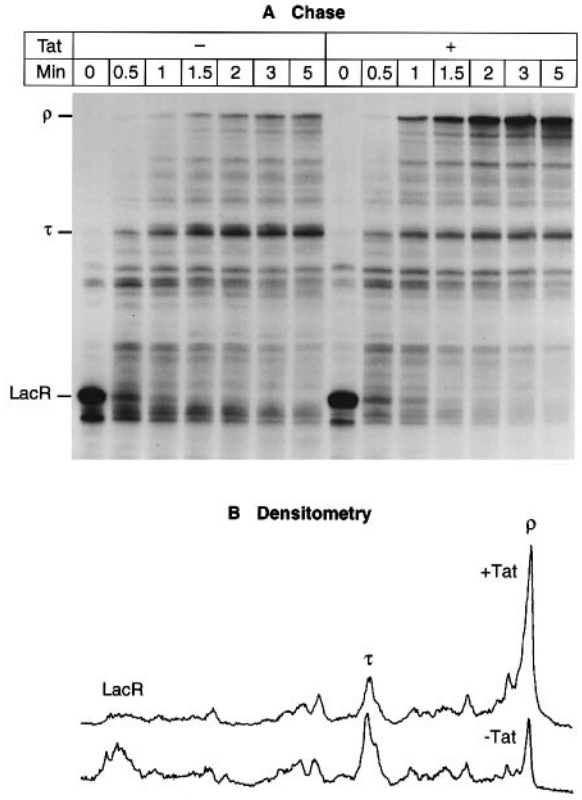


Fig. 3. Tat stimulates elongation by reactivated transcription complexes. (**A**) Transcription reactions were performed using immobilized template DNA carrying a wild-type TAR element in the absence (–) or presence (+) of 200 ng of recombinant Tat protein. After incubation for 20 min with [α-^{32}P]UTP and 1 μg of LacR, the immobilized templates were purified and treated with RNase H and the RHX1 and RHX2 oligonucleotides as described in the legend to Figure 2. The purified complexes were then 'chased' with 500 μM unlabelled NTPs in the presence of 100 μM IPTG. Aliquots were removed at the indicated times (0, 0.5, 1, 1.5, 2, 3 and 5 min) and the transcripts analysed by PAGE. (**B**) Quantitative analysis of the transcript distribution following 3 min of chase by densitometry. Note that, in the presence of Tat, there are significantly more transcripts found at ρ than in the absence of Tat. Similarly, in the absence of Tat, there are significantly more transcripts at τ than in the presence of Tat.

'pre-chase', essentially all of the labelled nascent chains are associated with polymerases that have been arrested by LacR.

Tat promotes formation of an activated transcription elongation complex

The experiment shown in Figure 3 demonstrates that transcription complexes arrested by LacR and purified on the streptavidin-coated beads, as described above, retain their capacity to elongate after the addition of NTPs. When LacR was dissociated from the templates by addition of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), and the paused polymerases are then 'chased' in the presence of unlabelled NTPs, the majority of the labelled transcripts become extended.

The processivity of transcription complexes prepared in the presence of Tat is significantly greater than that of complexes prepared in the absence of Tat. The densitometry patterns shown in Figure 3B (corresponding to the 3 min time point) demonstrate that 2.3-fold more polymerases reach the end of templates in the presence of Tat than in its absence. By contrast, there are ~1.5-fold more transcripts terminating at τ in the Tat-minus reactions than in the Tat-plus reactions. Another way to analyse the data is to compare the ratio of transcripts ending at ρ and τ. In the presence of Tat, more transcripts reach the end of the template than pause at the terminator, and $\rho/\tau = 1.95$, whereas in the absence of Tat, more transcripts pause at the terminator than reach the end of the template, and $\rho/\tau = 0.65$.

It is important to note that chase experiments of this design provide a direct measure of the elongation capacity of the paused polymerases. Since no new label is added to the reaction, all of the extended transcripts must have arisen by incorporation of unlabelled nucleotides at the 3′ ends of the pre-initiated transcripts. Furthermore, there is no background of transcripts arising from new initiations at the promoter. In contrast to continuous label experiments, where the extent of labelling of a transcript is proportional to its length, all of the labelled transcripts produced in the chase experiments have equivalent specific activities, and a direct comparison of the molar amounts is possible.

Activation of the transcription complex requires recruitment of Tat by a functional TAR RNA element

Tat-activated transcription in the cell-free system is strictly dependent upon the presence of a functional TAR RNA element. In previous studies, we have shown that when TAR is inactivated by either the G_{26}:C_{39}→C:G (mGC) mutation in the Tat-binding site or by the $G_{32–34}$→UUU (mLG) mutation in the apical loop sequence, the number of full-length transcripts produced in the presence of Tat is drastically reduced compared with templates carrying wild-type TAR elements (Graeble *et al.*, 1993; Churcher *et al.*, 1995; Rittner *et al.*, 1995; Keen *et al.*, 1996). The mGC mutation reduces Tat binding to TAR RNA by >14-fold and inactivates the viral LTR in reporter systems, whereas the mLG mutation does not affect Tat binding but prevents efficient *trans*-activation, presumably by preventing the binding of a cellular co-factor to TAR RNA (Churcher *et al.*, 1993).

To test whether the enhancement of elongation by Tat described above requires a functional TAR element, chase experiments were performed using templates carrying the mLG mutation in the TAR apical loop sequence. As shown in Figure 4, paused transcription complexes prepared using the mLG template do not respond to Tat. The pattern of transcripts produced during the chase experiment using the mLG template closely resembles the pattern seen for the wild-type template in the absence of Tat, with the majority of the transcripts terminating at τ and failing to reach ρ.

The protein composition of the stalled transcription complexes was analysed by immunoblotting (Figure 5). In agreement with our previous results (Keen *et al.*, 1996), a strong Tat signal was found in association with transcription complexes purified on templates carrying a wild-type TAR RNA element, but there is little or no Tat incorporated into transcription complexes formed on templates carrying mutant TAR elements or on templates where the HIV LTR (including TAR) has been replaced by the cytomegalovirus (CMV) promoter (Figure 5).

Technical improvements in the method, including the

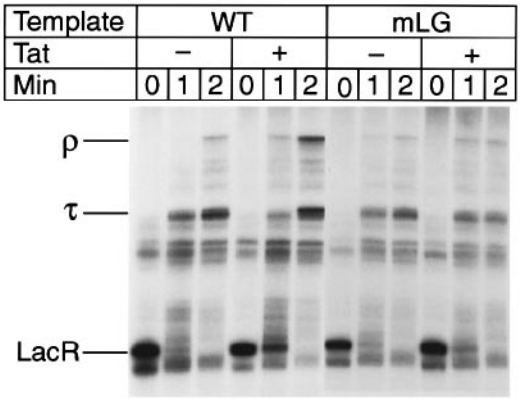


Fig. 4. Stimulation of transcription elongation by Tat requires an intact TAR element. Purified transcription complexes were prepared in the absence (–) or presence (+) of Tat protein as described in the legend to Figure 3. The template DNA carried either a wild-type TAR element (WT) or the mLG mutation in the apical loop of TAR (Churcher *et al.*, 1995; Keen *et al.*, 1996). The purified complexes were chased with unlabelled NTPs in the presence of IPTG, aliquots removed at the indicated times (0, 1 and 2 min) and the transcripts resolved by PAGE. Note that the mLG template produces a pattern of transcripts in the presence and absence of Tat that is similar to the wild-type template in the absence of Tat.

use of streptavidin–agarose beads and pre-bound templates, have reduced the background level of Tat considerably compared with our earlier experiments and permitted the high signal shown in Figure 5B. As in our previous experiments, measurements of RNA transcript levels (Figure 5A) and immunoblots for LacR and RNA polymerase II (Figure 5B) were used to provide an internal control for the recovery of proteins bound to the immobilized templates. As expected, the signals for LacR and RNA polymerase were essentially constant in this experiment.

In our previous study, restriction endonucleases were used to cleave the DNA templates downstream of the HIV LTR to release polymerases stalled at LacR (Keen *et al.*, 1996). In the experiment shown in Figure 5, the cleavage step was omitted, and proteins are detected both at the promoter and downstream of the promoter. No transcripts are produced in the presence of α-amanitin; however, there is a substantial amount of RNA polymerase bound to the templates near the promoter. Even in the presence of α-amanitin, the polymerase becomes hyperphosphorylated due to the activity of DNA-bound protein kinases, and only the II_o form of the enzyme is detected (Figure 5). Since no Tat is associated with templates prepared in the presence of α-amanitin, this result demonstrates that Tat is unable to become stably attached to the phosphorylated form of the RNA polymerase unless there has been transcription through a wild-type TAR RNA.

TAR RNA is released from the transcription complex

The results described above demonstrate that polymerases exposed to Tat show enhanced processivity and that Tat forms an integral part of the activated transcription elongation complex. Is TAR RNA also tightly associated with the transcription complex?

To answer this question, we performed the experiment outlined in Figure 6. Transcription complexes carrying ^{32}P-labelled nascent chains were prepared, paused at LacR

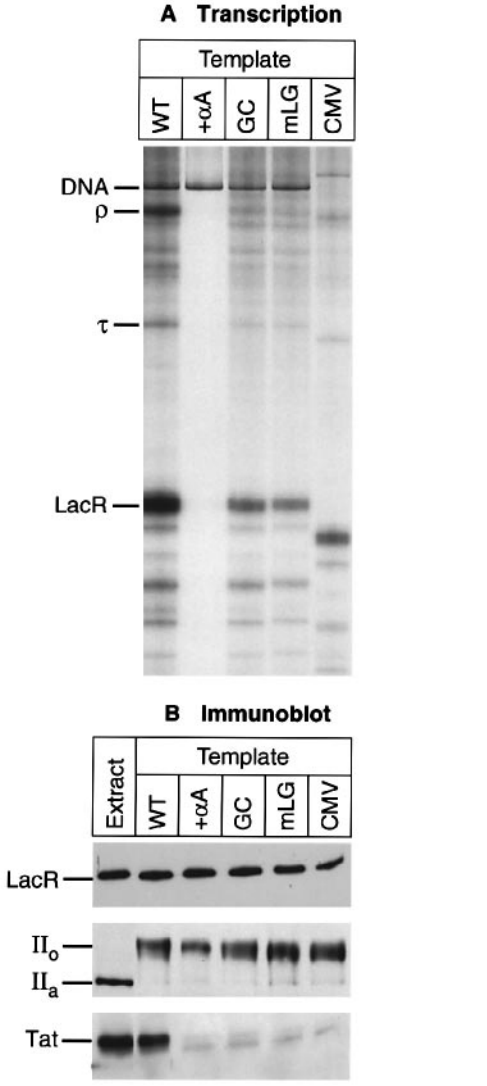


Fig. 5. Tat co-purifies with the arrested transcription complexes. (**A**) Transcription reactions were performed in the presence of 200 ng of Tat and 1 μg of LacR using immobilized template DNA carrying a wild-type TAR element. In parallel, reactions were performed in the presence of 1 μg/ml α-amanitin (+αA), or with templates carrying either the mGC or mLG mutations in TAR, or the CMV promoter replacing the HIV LTR (Keen *et al.*, 1996). After 20 min of labelling, the immobilized transcription complexes were purified, washed extensively, and the distribution of transcripts analysed by PAGE. To provide an internal control for the recovery of template DNA, the DNA was labelled with [α-^{35}S]dATPαS during the biotinylation reaction. Note that there is no active transcription in the presence of α-amanitin. (**B**) Analysis of the protein composition of the arrested transcription complexes by immunoblotting. Upper panel: LacR was detected using the NL1.1B2.10 antibody (Keen *et al.*, 1996). Middle panel: RNA polymerase II large subunit. The hyperphosphorylated (II_o) and hypophosphorylated (II_a) forms were detected using the 8WG16 antibody (Thompson *et al.*, 1990; Thompson and Burgess, 1996). Lower panel: Tat was detected using the antibody NT3.2D1.1 (Dingwall *et al.*, 1989). Note that Tat is only bound to the transcription complexes when a wild-type TAR element is present. Marker proteins corresponding to 5% of the proteins in the original extract are shown at the left of the figure (Extract).

and purified, as described above. The nascent RNA chains were then cleaved at distinct sites by hybridizing to DNA oligonucleotides and digesting the hybridized segment with RNase H. If TAR binds tightly to the transcription complex because of its interactions with proteins associ-

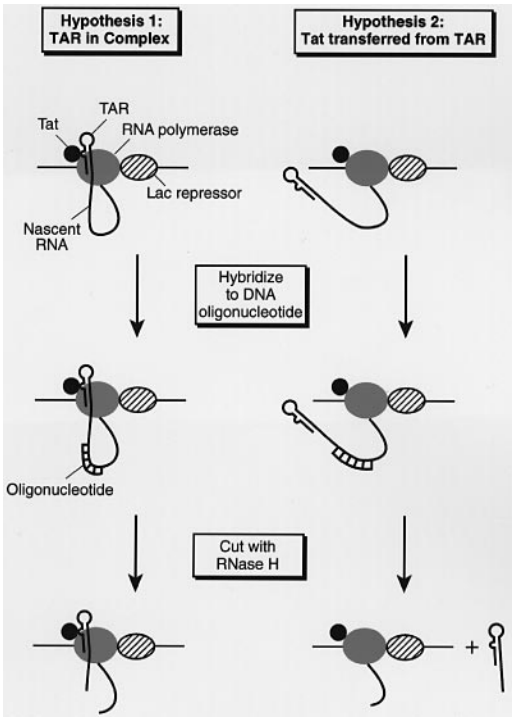


Fig. 6. Strategy used to determine whether TAR RNA is a component of the transcription complex (Hypothesis 1), or if Tat is transferred from TAR to the RNA polymerase complex (Hypothesis 2). Top: purified transcription complexes paused at LacR shown as either a ternary complex (Hypothesis 1) or with a free nascent RNA chain (Hypothesis 2). Middle: a DNA oligonucleotide which hybridizes 3′ to TAR is annealed to the nascent RNA chain. Bottom: the complex is digested with RNase H and washed extensively. If TAR RNA is tightly associated with the transcription complex (Hypothesis 1), then the 5′ end of the nascent chain, carrying TAR, will remain bound to the template DNA. Alternatively, if Tat is transferred from TAR to the polymerase (Hypothesis 2), then the 5′ TAR fragment will be released whilst the 3′ nascent RNA fragment will remain bound to the template DNA.

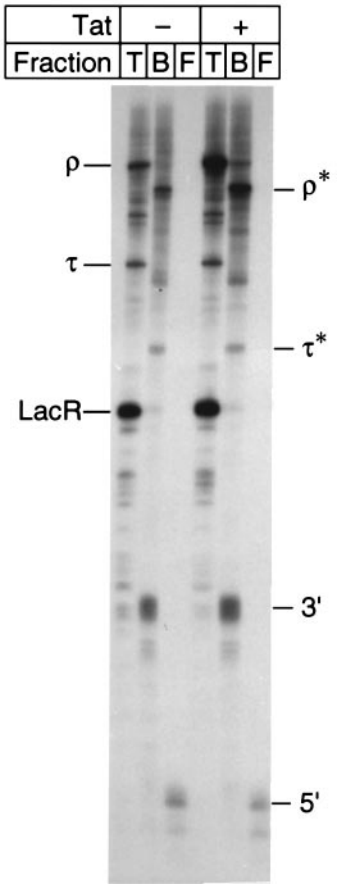


Fig. 7. TAR RNA is released from the transcription complex. Transcription reactions were performed using immobilized template DNA carrying a wild-type TAR element in the absence (–) or presence (+) of 200 ng of Tat protein. After incubation for 20 min with [α-^{32}P]UTP and 1 μg of LacR, the immobilized transcription complexes were purified and incubated with 1 μg of the DNA oligonucleotide RH7 (nucleotides 76–86) and RNase H. After 10 min, the beads were removed and the released free RNA (F) and the nascent chains bound to the immobilized complexes (B) were analysed by PAGE. An aliquot of the total undigested starting material (T) was used as a control. The positions of transcripts at the run-off (ρ), terminator (τ) and *lac* repressor (LacR) prior to cleavage are shown on the left. Cleavage by RNase H generates transcripts of reduced length at the run-off (ρ*) and terminator (τ*), a 103 nt fragment containing the 3′ end of the nascent RNA chain (3′) and a 75 nt fragment containing TAR RNA (5′). Note that the 3′ fragment is found exclusively in the bound fraction, whilst the 5′ fragment is quantitatively released.

ated with the polymerase, then the 5′ end of the nascent chain should remain bound to the transcription complex after cleavage by RNase H. On the other hand, if Tat is transferred from TAR during transcription elongation, then the 5′ end of the nascent RNA chain should be released.

As shown in Figure 7, cleavage of the nascent RNA with the oligodeoxynucleotide RH7 (complementary to nucleotides +76 to +86) and RNase H produces fragments of 75 and 104 nt, corresponding to the 5′ and 3′ ends of the nascent RNA chain. After purification of the complexes, the 3′ end of the nascent chain remains bound to the template, but the 5′ fragment carrying TAR is removed and recovered quantitatively in the free fraction. Since TAR RNA is also released when Tat is added to the reaction, the Tat protein does not serve as a bridging molecule that binds the 5′ end of the nascent chain to the RNA polymerase.

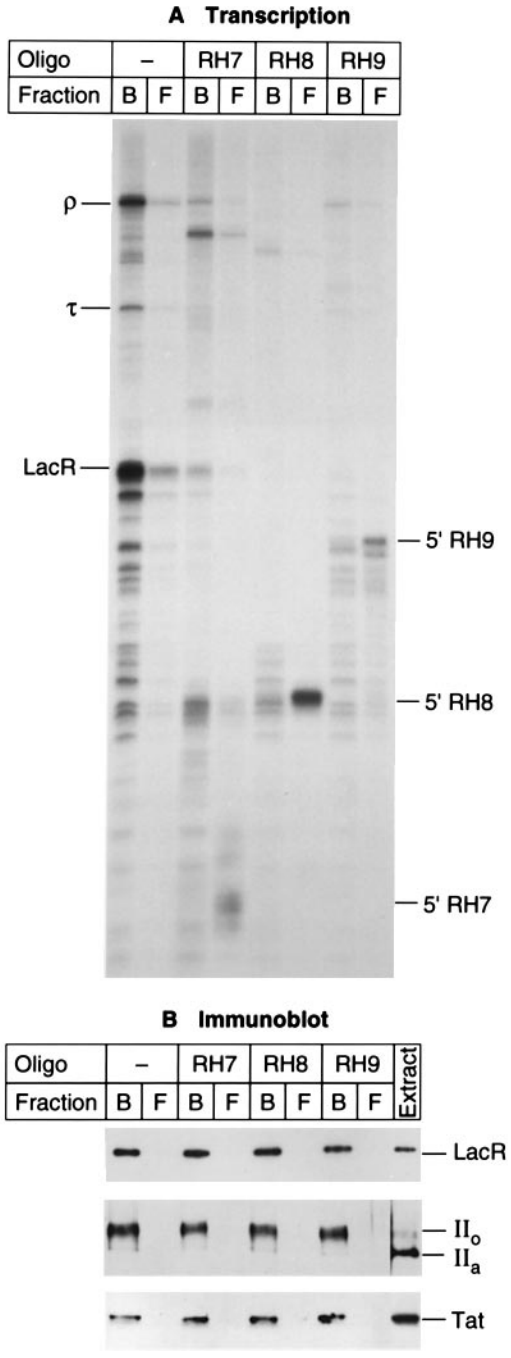
Similar results are obtained when the nascent chains are cleaved by RNase H following annealing to the DNA oligonucleotides RH8 (complementary to nucleotides +104 to +119) and RH9 (complementary to nucleotides +151 to +166) (Figure 8A). In both cases, the 5′ fragments carrying TAR (RH8, 104 nt and RH9, 151 nt) are quantitatively removed by washing. To demonstrate that Tat was not removed from the transcription complex following RNase H digestion, immunoblots were performed on half of the sample. As shown in Figure 8B, LacR, RNA polymerase II and Tat are all retained in the bound fraction.

Transcription complexes retain Tat activation in the absence of TAR RNA

If the major function of TAR is to provide a binding site for the loading of Tat and cellular co-factors on to the transcription complex, then removal of the 5′ end of the nascent chain should not affect the ability of Tat-activated transcription complexes to elongate. Chase experiments were therefore performed in which RH7 was included together with RHX1 and RHX2 during the RNase H digestion step. Figure 9 shows that when RH7 was present during the RNase H cleavage reaction, the length of the

nascent chain associated with polymerases paused at LacR was reduced by 75 nt (LacR*). During the chase, the polymerases resume transcription and the pre-labelled chains are extended. However, because the 5′ ends of the transcripts have been removed, the full-length products appear as chains reduced in length by 75 nt (ρ*). When Tat was present in the system, the polymerase exhibited enhanced processivity and significantly more polymerases were able to reach the end of template.

Thus, the paused transcription complexes remain transcriptionally active following the removal of TAR. There does not appear to be a requirement for TAR RNA to be continuously present in order to maintain the enhanced processivity of the Tat-activated transcription complexes. The simplest explanation for these results is that Tat is transferred from TAR to the transcription complex early in the reaction, probably during the transit of the polymerase through the TAR region.



Discussion

Transfer of Tat from TAR RNA to the activated transcription complex

The experiments described in this study exploit the observation that polymerases that have been arrested by the LacR protein remain bound to DNA and can be purified on templates attached to solid supports (Deuschle *et al.*, 1990; Kuhn *et al.*, 1990; Reines and Mote, 1993; Keen *et al.*, 1996). Even though the purified transcription complexes have been washed extensively, they still retain all the factors required for efficient transcription and can be reactivated by addition of NTPs.

Polymerases that have transcribed through TAR in the presence of Tat become stably modified and show significantly higher processivity than polymerases that have not been exposed to Tat. This activation of transcriptional elongation can be measured in 'chase' experiments as an increased ability of the polymerases to read through terminator sequences and reach the end of the template. It is important to note that these experiments have been designed to measure elongation in the absence of new initiations. Since no new label is added to the reaction during the chase, increases in the amount of full-length transcripts in the presence of Tat can only be due to an increase in polymerase processivity.

One obvious biochemical difference between the two types of transcription complexes is that Tat becomes tightly bound to the activated complex. Stable association of Tat with RNA polymerase requires transcription through a wild-type TAR RNA element. When transcription is blocked by the addition of α-amanitin, or TAR is mutated to inactivate either the Tat-binding site or the apical loop sequence, no Tat is recruited to the purified elongation complexes.

Tat is not tethered to the transcription complex via binding to TAR RNA, and the interactions between Tat and transcription complex are due to protein–protein interactions. Cleavage of the nascent chains present on the elongation complexes releases the 5′ end, containing the TAR region, but does not release Tat from the complex.

Fig. 8. Tat is retained as part of the transcription complex after removal of TAR RNA. (**A**) Transcription reactions were performed in the presence of 200 ng of Tat using immobilized template DNA carrying a wild-type TAR element. After incubation for 20 min in the presence of 1 μg of LacR and [α-^{32}P]UTP, the immobilized transcription complexes were purified from the reaction mixture and incubated with 1 U of RNase H and 1 μg each of the DNA oligonucleotides RH7 (nucleotides 76–86), RH8 (104–119) or RH9 (151–166). Control reactions were performed in the absence of added oligonucleotides (–). After 10 min digestion, the released, free RNA (F) was separated from the bound (B) fraction and analysed by PAGE. The positions of transcripts at the run-off (ρ), terminator (τ) and *lac* repressor (LacR) prior to cleavage are shown on the left. The positions of the 5′ cleavage products generated by RNase H cleavage following annealing to RH7 (75 nt), RH8 (104 nt) and RH9 (151 nt) are shown on the right. Note that the 5′ fragments, which contain TAR RNA, are always found in the free fraction. (**B**) Immunoblots of LacR, RNA polymerase II (II$_a$; II$_o$) and Tat from a parallel experiment (omitting the [α-^{32}P]UTP). Marker proteins corresponding to 5% of the proteins in the original extract are shown at the right of the figure (Extract).

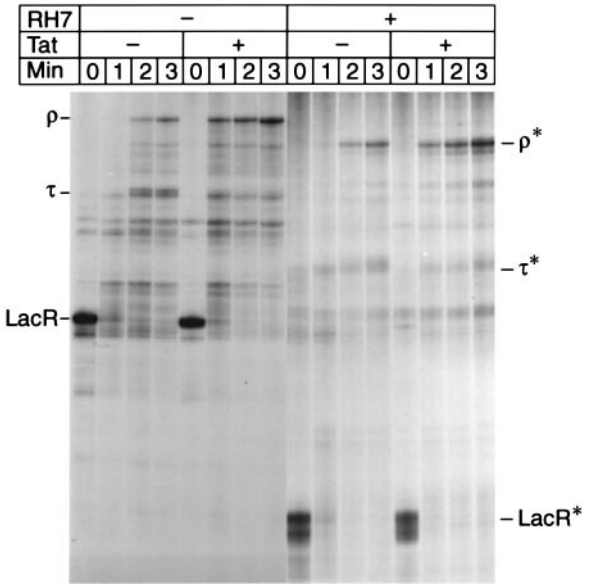


Fig. 9. Tat-activated transcription complexes show enhanced elongation capacity after removal of TAR RNA. Immobilized transcription complexes, enriched for transcripts ending at LacR using RNase H treatment and a pre-chase, were prepared as described in the legend to Figure 3. Reactions were performed in the absence (–) or presence (+) of 200 ng of Tat protein, 1 μg of LacR and [α-^{32}P]UTP. After washing to remove unbound RNA, the transcription complexes were chased with 500 μM unlabelled NTPs in the presence of 100 mM IPTG for the indicated times (0, 1, 2 and 3 min). In a parallel series of reactions, the oligonucleotide RH7 was included during the RNase H digestion to remove TAR RNA and produce a nascent RNA chain of 104 nt (LacR*). These complexes were then chased with 500 μM unlabelled NTPs in the presence of 10 mM IPTG. ρ* and τ* indicate the transcription run-off and termination products generated by chasing from the RNase H cleavage product LacR*.

Moreover, after the removal of TAR RNA, the transcription complexes still exhibit enhanced processivity when Tat is present. These results strongly suggest that TAR RNA is required only transiently to permit the efficient binding of Tat to the polymerase complex.

Recruitment of Tat to the transcription complex requires the synthesis of TAR RNA

There have been many attempts to demonstrate that Tat interacts with components of the transcription machinery prior to the synthesis of TAR RNA. Tat has been reported to bind to numerous proteins associated with transcription initiation, including TFIID (Kashanchi *et al.*, 1994; Veschambre *et al.*, 1995), Sp1 (Jeang *et al.*, 1993), TFIIH (Parada and Roeder, 1996), TAFII-55 (Chiang and Roeder, 1995) and TFIIB (Kashanchi *et al.*, 1994; Veschambre *et al.*, 1995). Unfortunately, because Tat is a 'sticky' protein, it has been difficult to demonstrate that its interactions with other proteins are specific and required for the activation of transcription.

In our experiments, we have only been able to detect a strong association between Tat and the transcription complex after the synthesis of a wild-type TAR element. Tat does not bind to polymerases that have transcribed through mutant TAR elements. Similarly, there is only negligible association of Tat with the RNA polymerase stalled near the promoter by the addition of α-amanitin (Figure 5) or when the reactions are performed in the absence of nucleotides (Keen *et al.*, 1996). It seems likely that the background of non-specific binding of Tat to components of the transcription apparatus was reduced under our experimental conditions because the binding reaction was performed in unfractionated HeLa cell extracts where there is competition for binding of the polymerase between Tat and all the other nucleic acids and transcription factors. By contrast, none of the protein interaction experiments suggesting that Tat may interact with components of the basal transcription apparatus were performed in the presence of competing TAR RNA or template DNA.

Our results strongly suggest that Tat is recruited to the elongation complex during the transcription of TAR. However, it should be noted that in our experiments we have only studied polymerases that are engaged in transcription and we have not analysed the composition of the pre-initiation complex. Tat can bind directly to hypophosphorylated RNA polymerase via its basic RNA-binding domain (Mavankal *et al.*, 1996). Similarly, Tat co-purifies with the RNA polymerase holoenzyme isolated from transfected cells (Cujec *et al.*, 1997). Tat can also be detected in association with pre-initiation complexes (García-Martínez *et al.*, 1997a). Although we think it is unlikely, our results do not exclude the possibility that Tat associates initially with the hypophosphorylated form of the RNA holoenzyme during transcription initiation and then dissociates from the hyperphosphorylated form of the enzyme during transcription elongation unless TAR RNA has been synthesized.

Trans-activation mechanism

The data described here suggest that recruitment of Tat and activation of elongation involves a series of sequential events, as shown in Figure 10. In common with most eukaryotic promoters transcribed by RNA polymerase II, the HIV LTR includes multiple upstream DNA regulatory elements which serve as binding sites for cellular transcription factors. The core promoter, which is functional in the cell-free transcription system, contains three tandem Sp1-binding sites (Jones *et al.*, 1986; Garcia *et al.*, 1987), a TATA element (Garcia *et al.*, 1989; Berkhout and Jeang, 1992; Olsen and Rosen, 1992) and a specialized initiator (INR) sequence (Jones *et al.*, 1988; Zenzie-Gregory *et al.*, 1993; Rittner *et al.*, 1995). Each of these elements is required for efficient initiation of transcription, but mutations in these elements do not permit initiation of transcription by an elongation-competent RNA polymerase (Rittner *et al.*, 1995). It therefore seems likely that initiation on the HIV LTR follows the normal pathway.

Early events regulating clearance of the HIV promoter are not well understood. Several groups have proposed that phosphorylation of the CTD of RNA polymerase is an early step in transcription that can be regulated by Tat (Herrmann and Rice, 1995; Chun and Jeang, 1996; Okamoto *et al.*, 1996; Parada and Roeder, 1996). In support of this hypothesis, Herrmann and Rice (1995) have reported that Tat binds a novel protein kinase that is capable of phosphorylating the CTD of RNA polymerase II. Similarly, Parada and Roeder (1996) and García-Martínez *et al.* (1997b) have shown that Tat can stimulate the kinase activity of purified TFIIH.

Unfortunately, our results suggest that CTD phosphorylation by DNA-associated protein kinases is not regulated

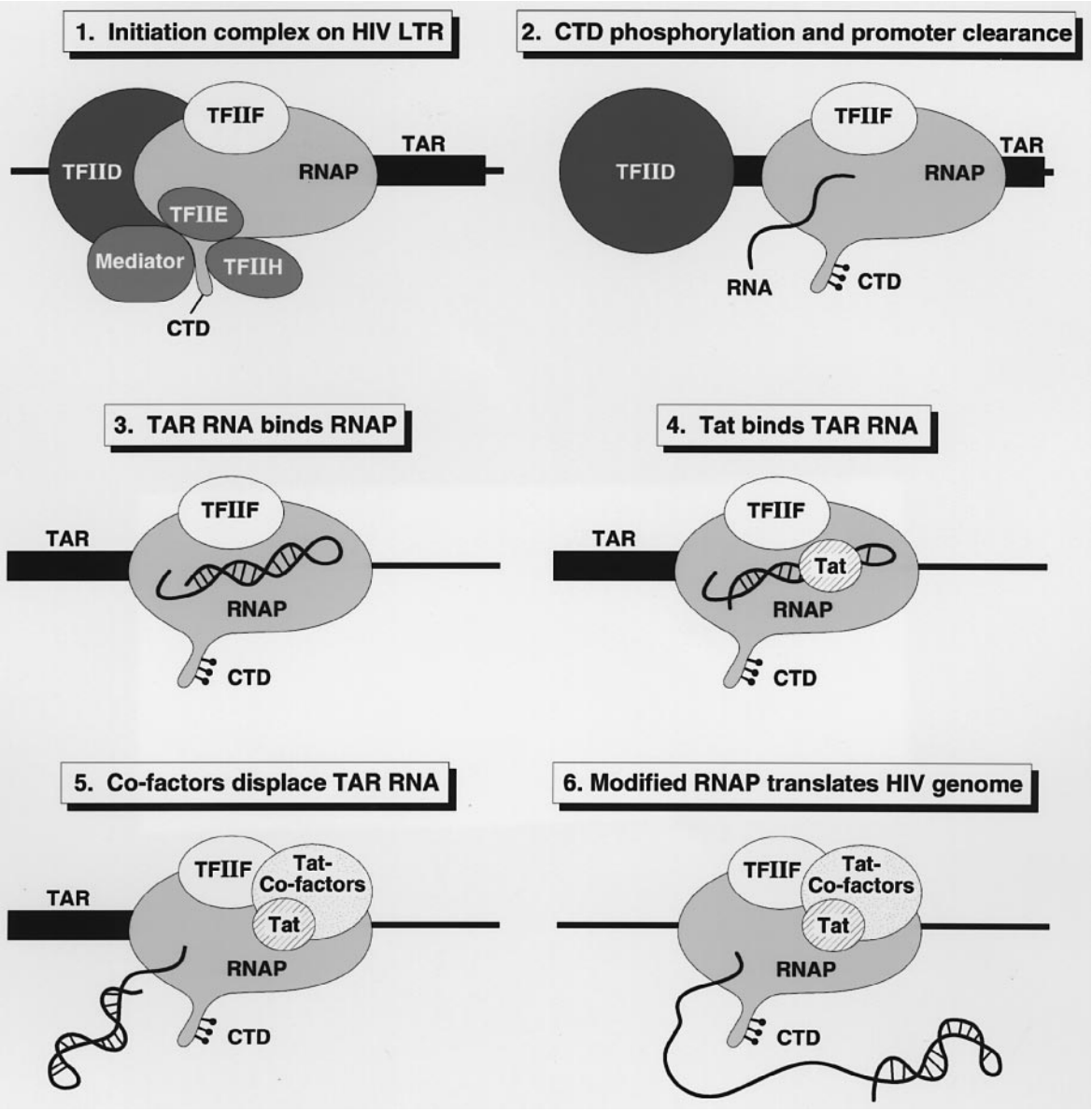


Fig. 10. Model for the activation of RNA polymerse II by Tat and cellular co-factors. Step 1: the RNA polymerase holoenzyme is recruited to the HIV LTR through its interactions with TFIID and other components of the basal transcription apparatus. Step 2: the CTD domain of the RNA polymerase is phosphorylated by TFIIH and the modified polymerase clears the promoter and begins transcription of TAR. Step 3: the TAR RNA transcript folds into its characteristic stem–loop structure and binds the RNA polymerase. The binding of TAR RNA to the transcription complex may result in a kinetic pause in polymerase elongation; however, TAR does not act as a terminator sequence and does not induce RNA polymerase to dissociate from the template. Step 4: Tat is recruited to the transcription complex because of its ability to bind to the bulge sequence found near the apex of the TAR RNA structure. Step 5: TAR is displaced from the polymerase by cellular co-factors associated with Tat. It seems likely that co-factors recognizing the TAR RNA loop sequence participate in this reaction. Additional co-factors that interact directly with Tat are probably also involved. Step 6: the activated transcription complex is able to transcribe the remainder of the HIV genome. Transcription complexes that have been activated by Tat show enhanced elongation capacity and are able to read through a variety of terminator sequences, including RNA stem–loop structures, with high efficiency.

by Tat. Prior to the addition of template DNA, the majority of the RNA polymerase II in the HeLa cell extract is hypophosphorylated (II_a, Figures 5 and 8). After binding to the template, the CTD is rapidly phosphorylated and quantitatively converted to the hyperphosphorylated form (II_o, Figures 5 and 8). We have found consistently that the polymerases become phosphorylated equivalently in the presence and absence of Tat (Figures 5 and 8, and data not shown). Since the two types of transcription complexes show distinct elongation properties in chase experiments, it seems unlikely that the change in processivity we have observed is due to selective CTD phosphorylation. In this context, it is important to note that Parada and Roeder (1996) have also been unable to demonstrate that Tat increases CTD phosphorylation of polymerases that are actively engaged in transcription. Their published data are consistent with our results, and show that when RNA synthesis is permitted the II_o form of RNA polymerase II is the predominant form of the enzyme detected both in the presence and absence of Tat. Thus, we believe that, in common with many other promoters (Laybourn and Dahmus, 1990; O'Brien *et al.*,

1994; Marshall *et al.*, 1996), CTD phosphorylation is an early rate-limiting step associated with the clearance of the promoter that takes place prior to the acquisition of Tat by the elongating polymerase. The phosphorylated polymerase is then able to transcribe through the TAR region and become additionally activated.

During the transcription of the TAR region, the TAR RNA stem–loop structure present on the nascent RNA strand is probably displayed on the surface of RNA polymerase. RNA polymerase II contains a binding site for the nascent RNA chain (Gu *et al.*, 1996). Furthermore, *in vitro* experiments have shown that RNA polymerase II is able to bind directly to TAR RNA (Wu-Baer *et al.*, 1995b). Although the sequence requirements for TAR RNA binding to RNA polymerase II have not been defined in great detail, the available evidence suggests that the apical loop sequence of TAR RNA participates in this reaction (Wu-Baer *et al.*, 1995b). Tat is then recruited to the transcription complex because of its ability to bind TAR RNA (Dingwall *et al.*, 1989).

Binding of Tat is known to induce a conformational change in TAR RNA (Puglisi *et al.*, 1992, 1993; Aboul-ela *et al.*, 1995, 1996; Brodsky and Williamson, 1997), and this in turn might create a recognition site for a cellular co-factor recognizing the TAR apical loop. Several proteins that recognize sequences in the loop have been identified (Sheline *et al.*, 1991; Wu *et al.*, 1991). The best characterized of these proteins is TRP185, which interacts with an extended region on TAR RNA that includes both the loop and the bulge region recognized by Tat (Wu *et al.*, 1991; Wu-Baer *et al.*, 1995a,b, 1996). TRP185 binding to TAR is incompatible with Tat binding to TAR, and the two proteins compete for TAR RNA in a concentration-dependent manner (Wu *et al.*, 1991; Wu-Baer *et al.*, 1995a). TRP185 is unable to bind TAR RNA on its own, but forms a tight complex with TAR in the presence of three additional protein co-factors (Wu-Baer *et al.*, 1996).

We propose that the loop recognition factors could help to dissociate Tat from TAR RNA, displacing the 5′ end of the nascent chain and permitting Tat to bind tightly to the RNA polymerase II. Thus, when an inappropriate loop sequence is present, the transfer reaction is blocked and Tat is unable to form a tight association with the transcription complex. Consistent with this hypothesis, we have shown here that mutations in the apical loop sequence (mLG) block both the recruitment of Tat to the elongation complex and the concomitant increase in polymerase processivity.

Once the exchange reaction has taken place, the activated polymerase is able to transcribe the remainder of the HIV genome efficiently. In addition to the cellular co-factors that recognize the TAR RNA loop, it seems likely that cellular co-factors that modify the polymerase processivity are also recruited to the Tat–RNA polymerase complex.

There is a growing body of evidence that protein kinases can regulate transcription elongation. For example, the positive transcription elongation factor b (P-TEFb), functions as both a kinase and an elongation factor (Marshall *et al.*, 1996). Protein kinases are also required for efficient *trans*-activation of the HIV LTR. In an early study, Jakobovits *et al.* (1990) reported that *trans*-activation of the HIV LTR by Tat could be blocked by protein kinase inhibitors, including H7. However, the identity of the kinase required for the Tat response is unclear, and several different enzymes that can be activated by Tat have been reported (Rice and Mathews, 1988; Herrmann and Rice, 1995; Parada and Roeder, 1996; Yang *et al.*, 1996; Zhou and Sharp, 1996; García-Martínez *et al.*, 1997b).

Conclusive evidence that kinases are used to regulate Tat-mediated transcription elongation will also require the identification of the physiologically relevant substrate. As described above, we believe that it is unlikely that phosphorylation of the CTD of RNA polymerase II is regulated by Tat. However, it is quite possible that other components of the transcription complex are phosphorylated by Tat-dependent kinases. A strong candidate for this role is pp140, a soluble cellular co-factor for Tat identified by reconstitution of a fractionated cell-free transcription system (Zhou and Sharp, 1995, 1996). pp140 has many of the attributes of a Tat-associated co-factor: it is required for an efficient *trans*-activation response, it binds Tat and it becomes phosphorylated by a kinase that can be stimulated by Tat. However, it remains to be demonstrated whether pp140 participates directly in the *trans*-activation mechanism or whether, like CTD phosphorylation, pp140 phosphorylation is an event that takes place prior to the transcription of TAR and the acquisition of Tat. A detailed comparison of the composition and phosphorylation state of pre-initiation complexes formed on the HIV LTR and of transcription complexes actively engaged in elongation should help to identify the functionally relevant co-factors for Tat.

Materials and methods

Template DNA

Plasmids carrying the wild-type HIV-1 LTR (p10SLT), the mGC mutation in the Tat-binding site of TAR (G_{26}:C_{39}→C:G, pGSLT), the mLG mutation of the apical loop of TAR ($G_{32–34}$→UUU, pMLT) or the CMV immediate-early promoter cloned upstream of TAR (pCMT) have been described elsewhere (Keen *et al.*, 1996).

Biotinylation of template DNA

Templates were linearized with *Xba*I and biotinylated by using the Klenow fragment of DNA polymerase I (Boehringer Mannheim), 50 μM biotin-16-dUTP (Boehringer Mannheim), 50 μM dCTP, 50 μM dGTP, 1 μM dATP and 1 μCi of [α-^{35}S]dATPαS. Unincorporated nucleotides were removed by gel filtration through Sephadex G50 (Pharmacia).

Pre-binding of templates to streptavidin beads

Biotinylated template DNAs (0.2 μg) were bound to either 20 μl of streptavidin–agarose (Immuno-pure streptavidin–agarose, Pierce Immunochemicals) or to 50 μl of streptavidin-coated magnetic beads (Dynabeads M280 streptavidin, Dynal) in wash buffer [20 mM HEPES pH 7.9, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 10 μM $ZnSO_4$, 80 mM KCl, 10% glycerol, 0.05% NP-40] for 5 min at 30°C. Beads were then collected by brief centrifugation (agarose beads) or magnetic concentration and washed twice with 200 μl of wash buffer to remove the unbound template DNA.

Cell-free transcription reactions

Streptavidin beads carrying 0.2 μg of template DNA were added to 40 μl of reaction mixture containing 0.8 μg of non-specific competitor DNA (pUC12 linearized with *Bam*HI), 15 μl of HeLa cell nuclear extract, 50 mM KCl, 4 mM $MgCl_2$, 20 mM HEPES pH 7.9, 2 mM DTT, 10 μM phosphocreatine, 100 μg/ml creatine kinase, 1 μg of poly[d(I–C)] (Boehringer), 50 μM GTP, 50 μM CTP, 50 μM ATP, 5 μM UTP and 10 μCi of [α-^{32}P]UTP (400 Ci/μmol; Amersham). For unlabelled reactions, the [α-^{32}P]UTP was omitted and the concentration of UTP was increased to 50 μM. Where indicated, 200 ng of Tat protein

and/or 1 μg of LacR was added to the reactions. The proteins were purified as described previously (Rittner *et al.*, 1995; Keen *et al.*, 1996). Reactions were incubated for 20 min at 30°C and the immobilized templates collected by either centrifugation (agarose beads) or magnetic concentration. The immobilized templates were then washed five times with 200 μl of wash buffer before being resuspended in 50 μl of wash buffer. To analyse RNA transcripts, 50 μl of stop buffer (150 mM Na acetate, 0.5% SDS, 10 mM EDTA, 20 μg/ml tRNA) was added, followed by extraction with an equal volume of phenol, and precipitation with two volumes of ethanol. The nucleic acids were dissolved in 8 μl of RNA loading buffer [80% (v/v) formamide, 10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol], denatured at 95°C for 5 min and analysed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea and TBE buffer (90 mM Tris base, 89 mM boric acid, 2.5 mM EDTA).

RNase H digestion

Full-length RNA attached to the end of the immobilized templates was removed by RNase H cleavage. Immobilized templates (50 μl in wash buffer) were incubated for 20 min at 30°C with 0.5 μg each of the DNA oligonucleotides RHX1 (CTAGCCTCCGATAGTCAAAA, nucleotides 270–290) and RHX2 (TGCACACAATAGAGGACTGC, nucleotides 400–420), 1 U of RNase H (Boehringer) and 40 U of RNasin RNase inhibitor (Promega). The immobilized templates were then collected by magnetic concentration and washed once with 500 μl of wash buffer to remove the cleaved RNA, before being resuspended in 50 μl of wash buffer prior to the chase reactions.

To remove TAR RNA, the templates were incubated with 1 μg of the oligodeoxynucleotide RH7 (GGCAAGCTTTA, nucleotides 76–86), RH8 (AACAGACGGGCACAC, nucleotides 104–119) or RH9 (ACACTG-ACTAAAAGG, nucleotides 151–166) and RNase H treated as above. To separate released RNA from bound RNA, beads were pelleted by brief centrifugation and the supernatant removed (free RNA). The pellet was washed twice in 200 μl of wash buffer before being resuspended in 50 μl of wash buffer (bound RNA) and processed for urea or SDS gel electrophoresis as described.

Chase experiments

RNase H-treated, immobilized transcription complexes were resuspended in 50 μl of wash buffer and 5 μl of chase buffer (5 mM ATP, 5 mM CTP, 5 mM GTP, 5 mM UTP and 100 mM IPTG). The reaction was incubated at 30°C for the indicated times (up to 5 min). Transcription was terminated by the addition of 50 μl of stop buffer before being processed for electrophoresis as described above.

Immunoblots

Samples for immunoblotting were denatured in SDS loading buffer (50 mM Tris–HCl pH 6.8, 100 mM β-mercaptoethanol, 1% SDS, 0.1% bromophenol blue, 10% glycerol) at 95°C for 5 min, fractionated by SDS–PAGE and transferred by electroelution to polyvinylidenediflouride membrane (Immobilon-P, Millipore). Tat was detected by the monoclonal antibody NT3.2D1.1 (Dingwall *et al.*, 1989), LacR by the monoclonal antibody NL1.1B2.10 (Keen *et al.*, 1996) and RNA polymerase II by the anti-CTD monoclonal antibody 8WG16 (a gift from N.E.Thompson) (Thompson *et al.*, 1990; Thompson and Burgess, 1996). Complexes were visualized with the ECL detection system (Amersham) by using rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (Dako).

Kinase labelling

Transcription reactions were set up using template DNA immobilized on streptavidin–agarose essentially as described above, except that nuclear extract was used from both HeLa cells and HeLa/C63 cells which constitutively express HIV-1 Tat protein. The [α-^{32}P]UTP was omitted, the concentration of unlabelled UTP increased to 50 μM and the reactions contained 10 μCi of [γ-^{32}P]ATP (300 Ci/μmol; Amersham). Reactions were incubated for 20 min at 30°C and the immobilized templates collected by centrifugation. The immobilized transcription complexes were then washed five times with 200 μl of wash buffer before being resuspended in 50 μl of wash buffer and denatured in SDS loading buffer (50 mM Tris–HCl pH 6.8, 100 mM β-mercaptoethanol, 1% SDS, 0.1% bromophenol blue, 10% glycerol) at 95°C for 5 min. Samples were then resolved by SDS–PAGE through 5% polyacrylamide gels before being subjected to immunoblotting to visualize RNA polymerase II and exposure to X-ray film to visualize the ^{32}P-labelled proteins.

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