Tat, a novel regulator of HIV transcription and latency

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Immediately after HIV infects a cell, the virion RNA is copied into DNA and the proviral genome is transported to the nucleus and integrated into the host cell genome. Once integrated into the host chromosome, the HIV becomes subject to regulation by cellular transcription factors, as well as its own regulatory proteins. HIV transcription is controlled primarily by the *trans*-activator protein (Tat). Tat provides the first example of the regulation of viral gene expression through control of elongation by RNA polymerase II. In the absence of Tat, initiation from the LTR is efficient but transcription is impaired because the promoter engages poorly processive polymerases that disengage from the DNA template prematurely.

Regulatory circuits controlling HIV transcription

The ability of the HIV LTR to act as an inducible promoter was first described by Sodroski *et al*. (1985) who noted that the synthesis of reporter genes placed under the control of the viral long terminal repeat (LTR) was stimulated 200- to 300-fold in cells which had been previously infected by HIV. They reasoned that the induction, or "transactivation", of transcription was due to the presence of a novel *trans*-activating factor, which they named Tat.

The majority of investigators assumed initially that Tat interacts directly with the promoter to stimulate initiation because most of the known regulatory proteins that control gene expression are DNA-binding proteins that work at the level of transcription initiation (Sharp & Marciniak, 1989). However, the pattern of transcription from the HIV LTR suggested that Tat does not stimulate initiation. When cells are transfected by plasmids carrying the HIV LTR characteristically produce two populations of transcripts, a set of short non-polyadenylated RNAs that terminate near the promoter, and a set of full-length polyadenylated mRNAs (Kao *et al*., 1987). In the absence of Tat, the short transcripts predominate, whereas in the presence of Tat there is a dramatic increase in the levels of long transcripts (Kao *et al*., 1987; Kessler & Mathews, 1992; Ratnasabapathy *et al*., 1990; Toohey & Jones, 1989). Thus, Tat participates in a positive feedback mechanism that ensures high levels of HIV transcription following the activation of cells carrying HIV proviruses. Furthermore, the shift from short to long transcripts observed in these experiments strongly implies that Tat actives transcription elongation.

The Tat/TAR RNA interaction

Deletion analysis of the viral LTR showed that the genetic element responsible for Tat activity, the transactivation-responsive region (TAR), is located downstream of the initiation site for transcription nucleotides +1 and +59 (Figure 1). The location of TAR in a transcribed region was surprising, since it suggested that TAR could function as an RNA sequence rather than as a DNA element (Muesing *et al*., 1987). The idea that TAR functions as an RNA regulatory signal gained further support from the observation that the TAR RNA sequence forms a highly stable, nuclease-resistant, stem-loop structure. Mutations that destabilise the TAR stem by disrupting base-pairing abolish Tat-stimulated transcription (Selby *et al*., 1989).

The simplest explanation for the ability of Tat to stimulate transcription from viral LTRs that carry TAR elements is that Tat binds directly to TAR RNA. After purification of recombinant Tat by an improved method we were able demonstrate that Tat is able to specially recognize TAR RNA (Dingwall

et al., 1989). Subsequently, it was noted by Weeks *et al*. (1990) that synthetic peptides carrying the basic domain of Tat are also able to bind directly to TAR RNA.

Tat recognition of TAR requires the presence of a U-rich bulge near the apex of the TAR RNA stem and the G26:C39 base pair immediately above the bulge. TAR RNA mutations that affect the structure of the U-rich bulge abolish Tat binding. However, so long as Watson-Crick base pairing is maintained, the identity of most of the base pairs throughout the TAR stem does not appear to be an important requirement for Tat recognition.

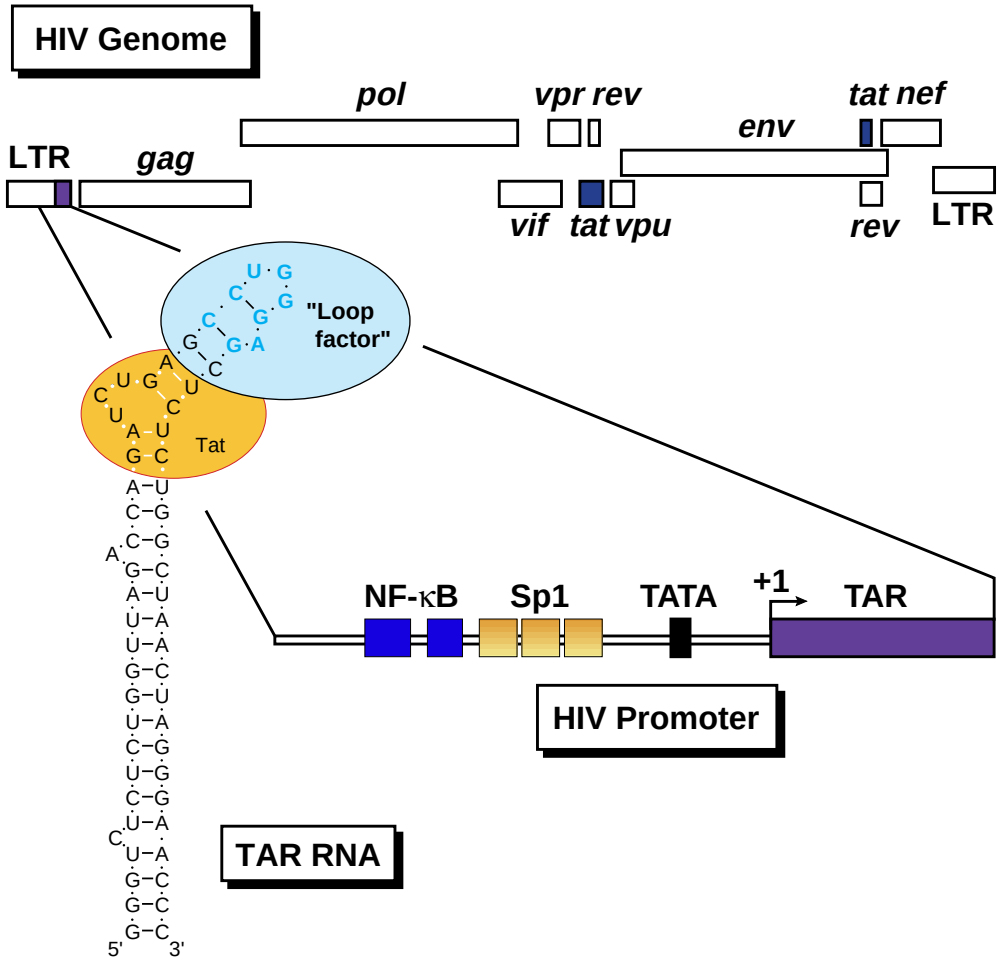

Figure 1. *Structure of the HIV genome (top), the viral promoter (bottom right) and TAR RNA (bottom left). The Tat gene is encoded by two exons that are highlighted on the HIV genome map. The viral promoter has a structure typical of promoters activated by RNA polymerase II. Immediately upstream of the TATA box are two tandem NF-κB binding sites and three tandem SP-1 binding sites. Immediately downstream of the start of transcription is the transactivation response region (TAR). TAR encodes an RNA that can fold into the stem-loop structure shown at left. Tat is able to bind directly to the highlighted region near to the apex of the stem containing a UCU bulge. Critical residues for Tat binding are shown in bold. In addition to acting as the binding site for Tat, TAR acts as the recognition signal for a cellular co-factor interacting with its apical loop sequence ("loop factor") (From (Karn, 1999)).*

Surprisingly, Tat recognition of TAR requires conformational changes in the RNA structure. NMR studies have shown that when Tat binds to TAR RNA the first residue in the bulge (U23) is displaced by one of the arginine side chains present in the basic binding domain of the Tat protein. The displaced U23 residue then forms an arginine binding pocket together with the critical G26:C39 base pair (Aboul-ela *et al*., 1995; Aboul-ela *et al*., 1996; Brodsky & Williamson, 1997; Puglisi *et al*., 1992). This conformational change also repositions critical phosphates on the TAR RNA backbone so that they then can be contacted by other basic residues found in the TAR RNA binding region and thereby ensure high affinity binding of Tat (Pritchard *et al*., 1994).

Although *in vitro* studies showed that Tat was a highly specific RNA binding protein, these studies did not by themselves prove that Tat recognition of TAR RNA occurred in the cellular context. In order demonstrate that this was actually the mechanism, it was necessary to correlate the in vitro binding of Tat to TAR with transactivation *in vivo*. The results showed that all the mutations that reduced the ability of TAR RNA to bind to Tat also blocked transactivation (Dingwall *et al*., 1990). Of particular significance was the observation that mutations that produced a partial reduction in Tat binding also produced a partial reduction in Tat response.

In vitro genetics

The first rigorous proof that Tat acts a general elongation factor became possible with the development of cell-free transcription systems that respond efficiently to Tat (Marciniak *et al*., 1990). Activation of transcription by Tat in the cell free system provides a remarkably accurate mimic of the *in vivo* mechanism. Studies using an extensive series of templates carrying mutations in TAR and the HIV promoter have shown that Tat-dependent transactivation in vitro has the same sequence requirements seen *in vivo* (Churcher *et al*., 1995; Rittner *et al*., 1995).

One of the most important results to emerge from studies using the cell free systems is the conclusive demonstration that Tat acts exclusively at the level of elongation. For example, Marciniak & Sharp (1991) reported that the distribution of RNA polymerases downstream of the viral LTR in the absence of Tat follows the pattern expected for a generalised elongation factor that uniformly reduces the probability of termination at each nucleotide along the template. A further experimental demonstration that exposure to Tat alters RNA polymerase processivity came from plasmids carrying artificial terminator sequences (Graeble *et al*., 1993). Finally, 'chase' experiments using elongation complexes paused during transcription by the *lac* repressor protein provided a formal demonstration that Tat constitutively activates the RNA polymerase (Keen *et al*., 1997). Transcription complexes that have transcribed through TAR in the presence of Tat showed high processivity, but complexes that were prepared in the absence of Tat or which had transcribed through mutant TAR elements showed defects in elongation.

The kinase connection

The constitutive modification of the transcription complex following exposure to Tat could be due to the recruitment of a transcription factor, or to the post-synthetic modification of the RNA polymerase, or a combination of both events. One likely modification of the RNA polymerase would be phosphorylation of the carboxyl terminal domain (CTD), since modification of the CTD is associated with promoter clearance (Laybourn & Dahmus, 1990; Marshall *et al*., 1996; O'Brien *et al*., 1994).

Rice and his colleagues (Herrmann *et al*., 1996; Herrmann & Rice, 1993; Herrmann & Rice, 1995) were the first to demonstrate that a protein kinase complex, which they called TAK (Tat-associated kinase) binds tightly and specifically to Tat. Cloning of the kinase subunit of TAK, the CDK9 kinase, by Zhu *et al*. (1997) revealed a homology between TAK and pTEFb, a positive acting elongation factor from Drosophila (Marshall *et al*., 1996; Marshall & Price, 1992; Marshall & Price, 1995). pTEFb is known to be present in early elongation complexes and required for elongation after promoter clearance.

Genetic and biochemical arguments have provided impressive evidence that TAK plays a critical role in the transactivation mechanism. First, it was shown that Tat-dependent transcription can be inhibited by protein kinase inhibitors such as DRB (Marciniak & Sharp, 1991) and a set of novel protein kinase inhibitors isolated from screens of drugs that inhibit HIV replication (Mancebo *et al*., 1997). Similarly, over-expression of CDK9 mutants *in trans* results in inhibition of TAK activity and the concomitant inhibition of Tat-dependent transcription (Gold *et al*., 1998; Mancebo *et al*., 1997; West & Karn, 1999). Finally, immunodepletion of the HeLa nuclear extracts using antibodies directed against CDK9 produced extracts that were unable to respond to Tat, but transactivation could be restored by addition of purified TAK (Mancebo *et al*., 1997; Zhu *et al*., 1997).

Since none of these experiments provided direct evidence that CDK9 is specifically activated by Tat during transcription the possibility remained that TAK simply plays an essential role in basal transcription. However, in recent experiments using the cell free system, we were able to show that Tat is able to stimulate additional CTD phosphorylation in elongation complexes and create a novel form of the RNA polymerase that we have called RNA polymerase II_o* (Isel & Karn, 1999). This phosphorylation event results in specific modifications of serine-5 in the CTD repeat (Zhou *et al*., 2000).

Regulation of CDK9 by cyclin T1 and TAR

In addition to carrying the kinase subunit CDK9, TAK also contains a cyclin subunit called cyclin T1 (Wei *et al*., 1998). Cyclin T1 is required for CDK9 kinase activity and promotes auto-phosphorylation of the C-terminus of CDK9 (Fong & Zhou, 2000; Garber *et al*., 2000; Garber *et al*., 1998a). Remarkably, in addition to regulating CDK9 activity, cyclin T1 is able to mediate Tat association with TAR RNA (Figure 2).

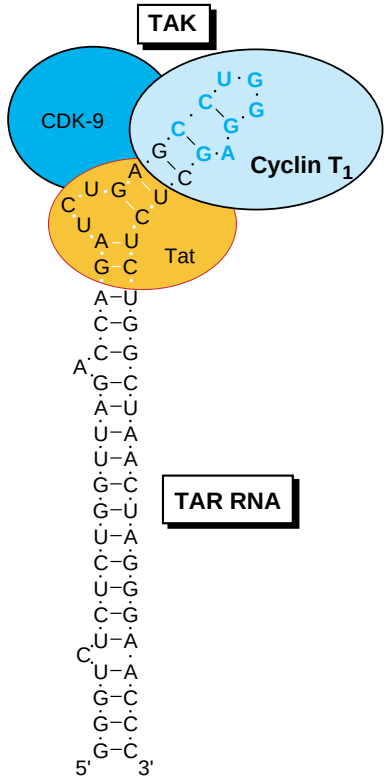

Figure 2. *Recognition of TAR RNA by Tat and TAK. Tat recognition primarily requires interactions with the bulge region of TAR. In the presence of cyclin T1, conformational rearrangements in Tat permit interactions with the apical loop sequences. Part of the interface between Tat and cyclin T1 is believed to involve cysteine residues from each protein that participate in zinc binding (From Karn, 1999).*

It has been known for many years that sequences in the apical loop of TAR RNA are not required for Tat binding even though this region of the RNA is essential for efficient *trans*-activation (Feng & Holland, 1988; Selby *et al*., 1989). The genetics implied that the loop acts as a binding site for cellular co-factors of Tat, however the identity of the loop factor remained mysterious and controversal for many years. The first unimpeachable evidence identifying the cellular co-factor required for loop recognition was obtained by Wei *et al*. (1998) who reported that cyclin T1, can form a stable ternary complex with Tat and TAR RNA that could be detected by gel electrophoresis. Ternary complex formation requires both the Tat binding site on TAR as well as the loop sequence. Similarly, Tat proteins carrying inactivating mutations, including truncations of the N-terminus, also failed to form the ternary complexes.

Genetic arguments were quickly assembled to confirm the identification of cyclin T1 as the "loop factor". Tat is mostly inactive in rodent cells even though initiation of transcription from the HIV LTR is efficient. This suggested that a critical cellular cofactor for Tat is dysfunctional in rodent cells. In keeping with this hypothesis, analysis of the cyclin T1 sequence found in murine cells showed that a critical cysteine residue found in the human cyclin T1 is replaced by a tyrosine (Bieniasz *et al*., 1998; Chen *et al*., 1999; Fujinaga *et al*., 1999; Garber *et al*., 1998b; Ivanov *et al*., 1999; Kwak *et al*., 1999). When the cysteine to tyrosine mutation is introduced into human cyclin T1, its ability to form a ternary complex with Tat and TAR RNA or to support HIV transactivation in transfected cells is blocked (Fujinaga *et al*., 1999; Garber *et al*., 1998b; Ivanov *et al*., 1999; Kwak *et al*., 1999). Thus, the mutation in cyclin T1 provides a molecular explanation for why rodent cells fail to support Tat-mediated transactivation even though initiation from the HIV LTR is efficient in these cells.

Structure of the Tat protein and its cofactors

Despite extensive efforts, the crystals of HIV-1 Tat have not been obtained. The best information about the three-dimensional structure of Tat is based on nuclear magnetic resonance (NMR) performed by (Bayer *et al*., 1995; Klostermeier *et al*., 1997; Metzger *et al*., 1997). These studies show that the Tat protein in isolation is largely unfolded.

The Tat sequence has been subdivided into several distinct regions on the basis of its amino acid composition: a N-terminal activation region (amino acids 1–19), a cysteine-rich role domain (amino acids 20–31), a core region (amino acids 32–47), a basic region (amino acids 48-57) and a glutamine-rich region (amino acids 60–76). Each of these regions is essential for Tat function. The role of the cysteine-rich domain is unclear, although it is believed to be involved in metal ion binding. The core, basic and glutamine rich region are all involved in RNA binding. In addition the basic region acts as a nuclear localisation signal. The most ordered parts of Tat in the NMR structure are composed of the core and glutamine-rich portions.

The structural basis for cyclin T1 interaction with Tat and TAR is still unclear. The most recent models suggest that Cyclin T1 does not bind to TAR directly, but it can alter the conformation of Tat to permit it to recognize the apical loop sequence of TAR RNA in the ternary complex. Cyclin T1 is believed to interact with Tat through metal ions stabilized by essential cysteine residues found in both proteins (Bieniasz *et al*., 1998; Garber *et al*., 1998b). Since the cysteine-rich and basic regions of the free Tat protein are highly flexible, it seems likely that they undergo conformational rearrangements during assembly of the ternary complex with cyclin T1 and TAR RNA. High resolution structures for the CDK9 kinase are also unavailable, however since CDK9 is highly homologous to the known CDK2 kinase structure it is possible to build a reasonably accurate model of the kinase domain. Clearly, a co-crystal structure of the Tat/TAK/TAR complex is badly needed!

The HIV LTR is a "defective" promoter

Although most studies of the HIV-1 transactivation mechanism have emphasised the role of Tat in stimulating transcription elongation, a key, but poorly understood, feature of this novel regulatory system is the ability of HIV-1 LTR to establish only non-processive (basal) transcription in the absence of Tat.

Does TAR act as an attenuator sequence? Alternatively, are there special features in the viral long terminal repeat that permit initiation by defective transcription complexes?

Surprisingly, extensive mutagenesis of the HIV-1 LTR has failed to identify any DNA elements that restrict elongation. If such an element existed, it would have been possible to delete this element and constitutively activate the HIV-1 LTR, however, all known mutations in the HIV-1 LTR simply reduce transcription initiation (Berkhout *et al*., 1990; Perkins *et al*., 1993; Rittner *et al*., 1995). Furthermore, there is no evidence that TAR, or any other downstream element acts as a specific attenuator sequence. If this were the case, then deletion of TAR would be expected to result in constitutively high expression of transcripts. Instead, TAR can be entirely deleted without changing the pattern of HIV transcription, provided that the normal transcription start site is left intact (Rittner *et al*., 1995). Thus, the defect in elongation appears to be an intrinsic feature of the promoter itself rather than due to a specific negative regulatory element.

Although elongation from the HIV LTR is defective, the promoter is able to initiate transcription very efficiently. In fact, the HIV LTR is able to support higher levels of transcription in HeLa cell extracts than either the adenovirus major late promoter or the CMV immediate early promoter. One reason why the HIV LTR is so efficient is that the core promoter, which includes all the elements required for transcription in the cell free systems, is optimally designed for cooperative binding by cellular transcription factors. The minimal functional HIV promoter requires only three tandem SP1 binding sites (Harrich *et al*., 1990; Jones *et al*., 1986), a TATA element (Garcia *et al*., 1989; Jones *et al*., 1988; Olsen & Rosen, 1992) and an initiator sequence (Zenzie-Gregory *et al*., 1993). Each of the core promoter elements participates in the co-operative binding of the initiation factor TFIID, a multi-subunit complex which contains the TATA binding protein, TBP, and co-factors called TAFs (Verrijzer *et al*., 1994). The TAFs can make direct contact with acidic activator proteins such as SP1 and the INR, while TBP recognises the TATA box.

The HIV-1 LTR can therefore be considered to be a “defective” promoter that lacks essential elements found in cellular promoters that promote elongation. Good candidates for this role are the enhancer elements. To test this hypothesis, we introduced the IgH enhancer into the HIV-1 LTR and showed that it is capable of constitutively activating a high level of Tat-independent transcription from the HIV-1 LTR in B-cells (West & Karn, 1999). In these experiments initiation levels in the presence or absence of the IgH enhancer were equivalent but only the transcription complexes that initiated in the presence of the enhancer were able to elongate efficiently (West & Karn, 1999). These results are consistent with those of Berkhout *et al*., (1990) and Chang *et al*., (1993), who showed that the addition of either the SV40 or the CMV enhancer to the HIV-1 LTR led to an increase in basal transcription and a reduced Tat response.

Transactivation mechanism

As we have seen, Tat permits activation of an unstable RNA polymerase that is engaged by the HIV core promoter. Because of its intrinsic instability, the polymerase either pauses or falls off the template soon after transcription through TAR unless Tat is present in the cell. Our current understanding of the molecular basis for the transactivation mechanism, based on the studies described above, is outlined in Figure 3.

During transactivation the RNA polymerase CTD is phosphorylated progressively by distinct enzymes. The kinase responsible for the phosphorylation in the pre-initiation complex has been identified as CDK7, which is a component of the TFIID initiation factor. Thus, in common with many other promoters (Laybourn & Dahmus, 1990; Marshall *et al*., 1996; O’Brien *et al*., 1994), CTD phosphorylation at the HIV LTR 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.

Following clearance of the promoter, the phosphorylated polymerase is then able to transcribe through the TAR region. During the transcription of the TAR region, the TAR RNA stem-loop structure is synthesized, and this creates a signal for Tat recruitment to the transcription complex. The interaction

between Tat and TAR not only involves the binding of Tat to TAR RNA but also its association with the protein kinase complex, TAK.

In the presence of Tat, the CDK9 kinase present in TAK becomes constitutively activated. Since the loop region of TAR is required for the formation of a ternary complex between Tat, TAR RNA and cyclin T1, it seems likely that conformational changes in TAR RNA and cyclin T1 which are associated with Tat binding, alter the conformation of CDK9 and thereby activate the enzyme. Activation of the CDK9 kinase, in turn leads to CTD hyperphosphorylation and the creation of the II_o* form of the RNA polymerase (Isel & Karn, 1999).

Modification of the transcription complex by Tat and TAK to make it more processive may also require other protein phosphorylation events in addition to the modification of the RNA polymerase CTD. Purified TAK is able to auto-phosphorylate resulting in the modification of both CDK9 and cyclin T1 (Mancebo *et al*., 1997; Wei *et al*., 1998; Zhu *et al*., 1997). The phosphorylation reaction also promotes the interactions between Tat, TAK and TAR RNA (Fong & Zhou, 2000; Garber *et al*., 2000; Ivanov *et al*., 1999; Zhang *et al*., 2000). In addition, Wada *et al*. (1998) have shown that TAK can be used to reverse

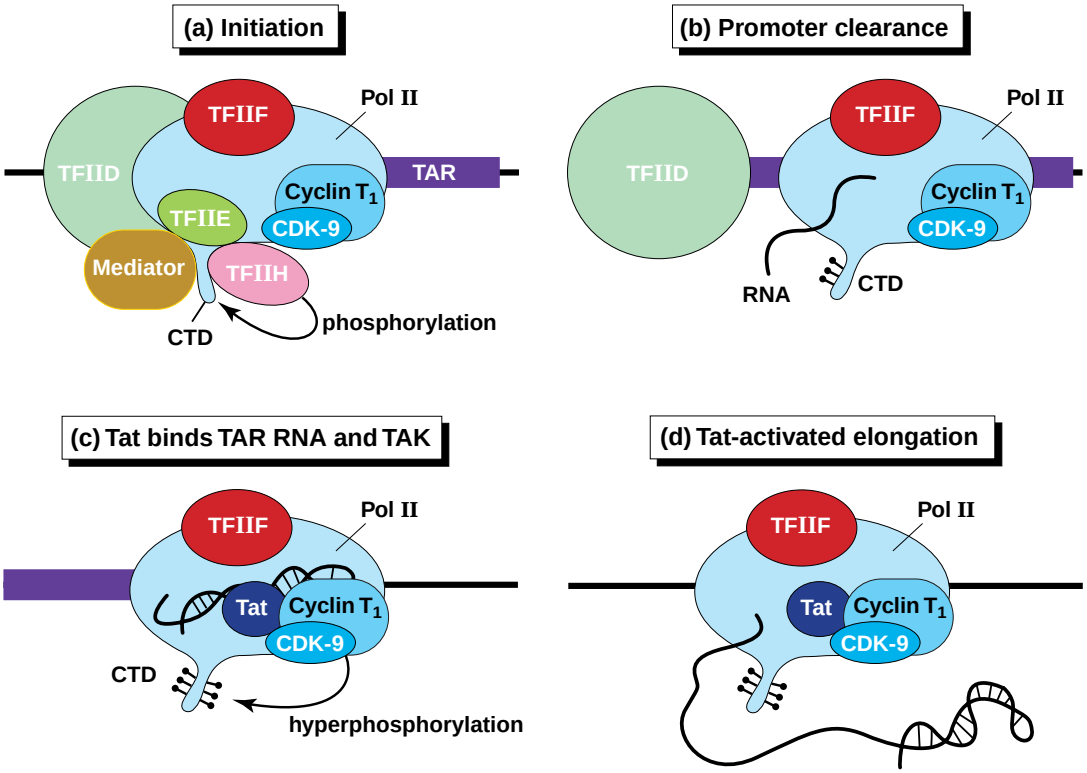

Figure 3. *Model for the activation of RNA polymerase II by Tat and cellular co-factors. (a) The RNA polymerase II holoenzyme is recruited to the HIV LTR through its interactions with TFIID and other components of the basal transcription apparatus. The CTD domain of the RNA polymerase is phosphorylated by the CDK-7 kinase found in TFIIH and the modified polymerase clears the promoter and begins transcription of TAR. (b) The nascent RNA chain corresponding to the TAR RNA transcript folds into its characteristic stem-loop structure and binds the RNA polymerase. (c) 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 and forms a ternary complex with TAK. The activated TAK kinase catalyses phosphorylation of the CTD and TAR is displaced from the polymerase. (d) The activated transcription complex is able to transcribe the remainder of the HIV genome. (From Karn, 1999).*

an early negative effect on transcription by the DRB-sensitivity inducing factor (DSIF), which is composed of the transcription factors Spt4 and Spt5. Spt5 is also an essential cofactor for Tat-activated transcription (Ivanov *et al*., 2000; Wu-Baer *et al*., 1998). Very recent studies have shown that Spt5 is also a substrate of Tat-activated CDK9 (Kim & Sharp, 2001; Ping & Rana, 2001). How all these phosphorylation events are coordinated during transcription is the subject of much current research.

Control of latency

In rapidly replicating cells HIV is transcribed almost immediately. However, some infected T-cells subsequently enter quiescence, with the result that the integrated proviral genome enters a latent state where it can remain transcriptionally silent until the cell receives a mitogenic signal. The ability to infect cells latently helps HIV to establish persistent infections despite strong immune responses against the viral proteins. When T-cells residing in lymphoid tissues are activated, the latent proviruses that they harbor can efficiently infect adjacent cells through cell-to-cell transfer of virions (Grossman *et al*., 1998; Pantaleo *et al*., 1993). It is extremely difficult for antibodies to block this type of virus spread since free virus particles, which can be efficiently targeted are not released.

A second consequence of latency during the development of HIV disease is that the latent proviruses create a large and stable reservoir of genetic variants from which strains carrying resistance to immune responses and therapeutic drugs can be selected (Wei *et al*., 1995). Although most studies of the HIV-1 transactivation mechanism have emphasized the role of Tat in stimulating transcription elongation, a key, but poorly understood, feature of HIV growth is its ability to establish a latent infection where the promoter is entirely shut down, and then reactivate transcription, even in the absence of Tat.

What are there special features of the viral long terminal repeat that permit promoter shutdown and reactivation? In addition to the core promoter elements, described above, the HIV promoter contains two NF-κB binding sites located near the transcription start site. Studies using viral LTRs linked to reporter genes demonstrated that the transcription factor NF-κB plays a central role in the proviral activation pathway (Nabel & Baltimore, 1987). In resting T-cells and most established T cell lines, NF-κB is sequestered in the cytoplasm by the inhibitor protein IκBα(for reviews, see Baeuerle & Henkel, 1994 and Baldwin, 1996). Following exposure of T-cells to antigen, or treatment of the cells by mitogens such as phorbol myristyl acetate (PMA) and phytohaemaglutinin (PHA), NF-κB is unmasked due to the sequential phosphorylation, ubiquitination and degredation of IκBα. Free NF-κB then translocates to the nucleus where it can activate transcription from a wide variety of promoters, including the HIV LTR (Nabel & Baltimore, 1987; Perkins *et al*., 1993).

Although the critical role played by NF-κB during the activation of HIV transcription is well established, the precise molecular mechanisms underlying its activity are still largely unknown. Most models of HIV activation assume that the increased levels of NF-κB exclusively stimulate initiation rates from the viral LTR. According to these models, Tat is produced initially simply because the promoter is “leaky” and a small fraction of the transcription complexes are able to fortuitously produce full-length transcripts (Marciniak & Sharp, 1991). However, it seems more likely that rather than relying on a stochastic process, specialized mechanisms exist that ensure early Tat production.

Recent evidence from our laboratory (M. West and J. Karn, unpublished) suggests that NF-κB acts to stimulate both transcription initiation and, to a lesser degree, elongation (Figure 4). Following integration into the host chromosome, the HIV-1 provirus is assembled into chromatin, with nucleosomes that are positioned at precise locations around the viral promoter (Sheridan *et al*., 1997; Van Lint *et al*., 1996). The first nucleosome downstream of the start site (Nuc-1) impedes transcription by occluding the initiation site. There is strong evidence that Nuc-1 is disrupted following the activation of HIV-1 transcription by TNF-α and PMA in transfected T-cells and latently infected cell lines (Van Lint *et al*., 1996). This modulation of the chromatin structure appears to be mediated by recruitment of histone acetylases to the HIV LTR by NF-κB. Consistent with this idea, the histone deacetylase inhibitor Trichostatin A (TSA) can promote the hyperacetylation of histones and activate HIV-1 transcription initiation (Sheridan *et al*., 1997; Van Lint *et al*., 1996).

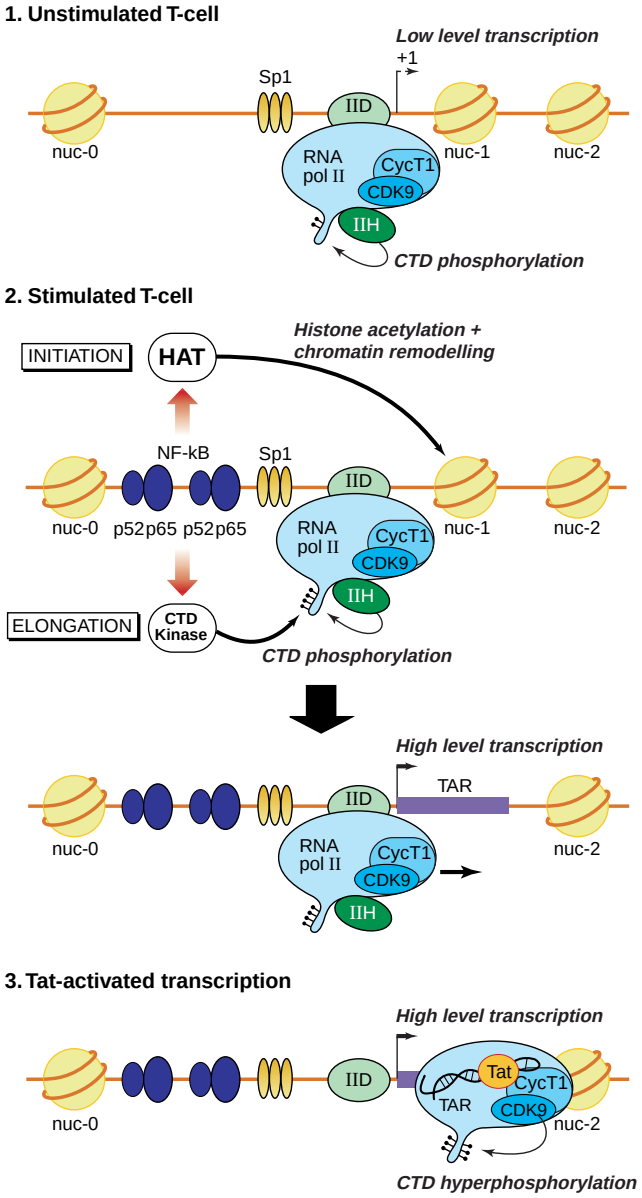

Figure 4. *Activation of latent proviruses by NF-κB and Tat during T-cell activation. (1) In unstimulated T-cells cells the HIV-1 proviral DNA is assembled into chromatin with precisely positioned nucleosomes surrounding the transcription start site. (2) After stimulation of T-cells by mitogens, NF-κB binds to its recognition sites in the LTR. NF-κB associates with a coactivator complex containing histone acetylases. These chromatin remodelling factors, leads to the disruption of Nuc-1 and the stimulation of transcription initiation. In addition, NF-κB stimulates the activity of a CTD kinase which catalyses the phosphorylation of the CTD of RNA polymerase II at the promoter. This results in the stimulation of transcriptional elongation. (3) Transcription from the activated LTR leads an initial round of Tat synthesis. (4) Tat binds to the TAR RNA element and further activates transcription by stimulating elongation.*

Our new observation is that NF-κB can also influence transcription elongation. RNase protection studies showed that the proportion of total transcripts extending to distal regions of the template was disproporationately increased following NF-κB expression. This demonstrates that DNA elements present in the LTR can regulate elongation and is consistent with our earlier observation that the insertion of a cellular enhancer into the LTR led to the recruitment of processive polymerases to this promoter and alleviated the requirement for Tat (West & Karn, 1999). One molecular mechanism consistent with the ability of NF-κB to stimulate elongation is its ability to stimulate phosphorylation of the RNA polymerase CTD at certain promoters. Nissen & Yamamoto, (2000) have shown that NF-κB can stimulate the phosphorylation of the CTD of the RNA polymerase II complexes assembled at the promoters of the IL-8 and ICAM-1 promoters.

The synergistic activities of NF-κB and Tat to stimulate HIV transcription acts as a "molecular switch" that allows HIV to make the transition from a latent state to lytic growth. In HIV-2 and SIV where only one NF-κB site is unusually present, additional regulatory proteins with enhancer like activities are probably used to augment early transcription. These cellular DNA-binding transcription factors can act as rate limiting steps for initiation of transcription, but ultimately, the rate of virus growth is moderated by the regulatory proteins. If the LTR activity is too weak to allow the production of necessary levels of Tat, or there are insufficient Tat levels to allow the threshold production of the viral proteins, virus replication will be aborted.

Extracellular Tat

In addition to playing its critical role as a transcription factor there is a growing body of evidence that Tat might contribute to HIV disease as an extracellular protein. Several groups have shown that Tat can be secreted by infected cells (Ensoli *et al*., 1994; Goldstein, 1996). Furthermore, extracellular Tat is readily taken up by cells through interactions with heparin sulfate proteoglycans displayed on the cell surface (Frankel & Pabo, 1988; Tyagi *et al*., 2001). Subsequently, Tat can reach their nucleus and then activate a variety of cellular genes including cytokines such as interlukin 6 (IL-6), IL-2 and tumor necrosis factor α (Ambrosino *et al*., 1997; Nath *et al*., 1999) and the CXCR4 and CCR5 cytokine coreceptors for HIV infection (Huang *et al*., 1998). Another potentially important target for exogenous Tat is the vascular endothelium where it is able to induce angiogensis and inflammation (Mitola *et al*., 2000; Rubartelli *et al*., 1998). Thus, there are many opportunities for Tat to act as a contributor to HIV pathogenesis in an autocrine or paracrine fashion.

Most of the effects of extracellular Tat have been observed in tissue culture cells exposed to recombinant Tat. Unfortunately, it is still not clear whether enough Tat is released during HIV infections to produce analogous effects *in vivo*. Some years ago, we tested a series of T-cell lines that constitutively expressed Tat introduced via a retroviral vector. Although these cells expressed many times the level of Tat that was required for transactivation, Tat was not released into the medium unless there was apoptosis. Until direct evidence from studies using animal models becomes available, it will remain uncertain whether extracellular Tat plays an important role in the development of HIV disease.

Novel targets for drug discovery

Current anti-viral treatment for HIV infections is restricted to inhibitors of reverse transcriptase and/or the viral protease. Although the available drugs effectively reduce the levels of rapidly replicating virus, the benefit is only short-term because of clinical side effects and the emergence of drug-resistant strains. Most resistance mutants that are detected in clinical trials pre-exist in HIV infected individuals, but because these mutations reduce virus growth rates, they will only emerge as dominant strains during drug treatment. Although multiple-drug combination therapy leads to the virtual removal of virus from the peripheral circulation, its efficiency in preventing resistance over long periods of treatment remains to be proven. A second problem in current HIV chemotherapy this that even when most viral replication

is inhibited, the population of latently infected cells remains unaffected. Stimulation of these cells by cytokines produces viruses that rapidly repopulate the body once therapy is interrupted (Embretson *et al*., 1993; Ho *et al*., 1995; Pantaleo *et al*., 1993; Temin & Bolognesi, 1993; Wei *et al*., 1995).

The complex mechanism regulating transcription of the HIV genome provide particularly attractive targets for anti-viral development since regulated transcription of HIV is required not only during the exponential growth of the virus, but also critically, during the activation of the integrated proviral genomes that give rise to the drug-resistant strains. Now that molecular studies of the activation of transcription by Tat and its cellular co-factors are nearing completion, there is renewed interest in finding drugs that selectively inhibit these processes. The search for drugs that can block transactivation started several years ago using a variety of cellular and in vitro screens.

The best known HIV transcription antagonists were a pair of benzyldiazepine discovered at Roche, called Ro5-3335 and Ro24-7429 (Cupelli & Hsu, 1995; Hsu *et al*., 1991). Detailed analysis of HIV-1 transcription using nuclear run on assays has shown that these compounds inhibit the initiation of HIV transcription but do not specifically interfere with Tat (Cupelli & Hsu, 1995). Ro24-7429 is the only transcription inhibitor to have progressed into Phase I clinical trials. Unfortunately, these trials were abandoned due to side effects of the drug on the central nervous system before antiviral activity in patients could be demonstrated. One contributing factor to the lack of antiviral activity may have been extensive binding of the drug to human plasma proteins (Cupelli & Hsu, 1995).

Several protein kinase C inhibitors have been shown to inhibit HIV LTR-driven gene expression by reducing NF-κB availability (Jakobovits *et al*., 1990). One of the most potent of these compounds is pentoxifylline (PTX) (Mhashilkar *et al*., 1997). Unfortunately, pentoxifylline inhibition of HIV is restricted to certain cell types and in clinical trials there is was no evidence found that the viral loads in patients treated with pentoxifylline were reduced (Dezube & Lederman, 1995).

The most selective inhibitors of HIV replication currently known are the fluoroquinolones such as K12 and K37 (Baba *et al*., 1998; Baba *et al*., 1997; Witvrouw *et al*., 1998). Unlike the Roche compounds, the fluoroquinolones appear to directly target a step in transactivation and are potent inhibitors of Tat-activated transcription under conditions where CMV transcription and basal transcription from the HIV LTR are unaffected. Unfortunately, although these compounds inhibit a broad range of viruses they are also cytotoxic and exhibit a narrow selectivity index. Until the molecular targets for these interesting drugs is identified, and the selectivity index improved, it is difficult to see how they can be progressed further.

A more selective approach is to identify protein kinases inhibitors that selectively inhibit CDK9 (TAK). It has been known for many years that protein kinase inhibitors such as DRB can selectively inhibit Tat-mediated transcription elongation (Marciniak & Sharp, 1991). The first successful screen for inhibitors of TAK was reported by Mancebo *et al*. who have used a screen of over 100,000 compounds in a Tat-dependent in vitro transcription assay to discover a diverse set of protein kinase inhibitors (Mancebo *et al*., 1997). Although TAK kinase inhibitors can effectively block HIV replication, it should be borne in mind that each of these compounds is also cytotoxic because TAK is also required for cellular transcriptional control (Chao *et al*., 2000; Flores *et al*., 1999). Once again, until the potency and selectivity indexes of these compounds are increased it is unlikely that these types of inhibitors can progress to development.

Finally, it may be possible to select small molecules that inhibit the Tat/TAR interaction. Using a combinatorial approach (Hamy *et al*., 1997) were able to identify a peptidic compound, CGP64222, that is able to effectively compete with Tat binding for TAR RNA and inhibit HIV replication. NMR analysis shows that CGP64222 binds directly to TAR RNA at the Tat binding site. Thus, CGP64222 is the first example of an antiviral compound that selectively inhibits a RNA-protein interaction. In later developments some promising non-peptidic inhibitors of the Tat-TAR interaction were also uncovered (Hamy *et al*., 1998; Mei *et al*., 1997).

Conclusions

The central role played by Tat in HIV replication has made it a focus of attention ever since its

discovery in 1985. Tat provides the first example of a protein that regulates transcriptional elongation in eukaryotic cells, and until Tat was discovered, elongation control was a relatively neglected area of transcription regulation. Although there is still a great deal of ignorance about the mode of action of the key elongation factors, the identification of CDK9, cyclin T1 and Spt5 as critical components of the Tat-dependent elongation mechanism has also revealed their importance in the normal cellular transcription.

Studies of the interactions of Tat with TAR RNA have also provided new insights into the chemistry of nucleic acid recognition. There are many examples of RNA binding proteins that recognise bases displayed in apical loop and distorted bulge structures. Studies of the Tat-TAR interaction have revealed that the binding reaction involves both a conformational changes in RNA structure that provides much of selectivity for molecular recognition.

Finally, studies of HIV transactivation are providing a strong molecular basis for drug discovery. Several groups have identified promising "hits" that inhibit HIV replication by interfering with Tat, TAR and cellular cofactors required for HIV transcription. As structural data on these molecular targets becomes available, further development of these compounds will become possible using modern structure-based design. I am optimistic that these existing early leads can be developed into drugs that will make a useful contribution to the treatment of AIDS.

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