Stimulation of Tat-associated kinase-independent transcriptional elongation from the human immunodeficiency virus type-1 long terminal repeat by a cellular enhancer

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The human immunodeficiency virus type-1 (HIV-1) long terminal repeat (LTR) initiates transcription efficiently but produces only short transcripts in the absence of the *trans*-activator protein, Tat. To determine whether a cellular enhancer could provide the signals required to recruit an elongation-competent polymerase to the HIV-1 LTR, the B cell-specific immunoglobulin heavy chain gene enhancer (IgHE) was inserted upstream of the LTR. The enhancer increased transcription in the absence of Tat between 6- and 7-fold in transfected B cells, but the full-length transcripts remained at basal levels in HeLa cells, where the enhancer is inactive. RNase-protection studies showed that initiation levels in the presence and absence of the enhancer were constant, but the enhancer significantly increased the elongation capacity of the polymerases. Tat-stimulated elongation is strongly inhibited by the nucleoside analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), which inhibits the Tat-associated kinase, TAK (CDK9). However, polymerases initiating transcription from LTRs carrying the enhancer were able to efficiently elongate in the presence of DRB. Specific repression of TAK by expression *in trans* of the CDK9 kinase also inhibited Tat-stimulated elongation but did not inhibit enhancer-dependent transcription significantly. Thus, the activation of polymerase processivity by the IgHE involves a unique mechanism which is independent of TAK.

Keywords: HIV/enhancer/RNA polymerase II/Tat/transcriptional elongation

Introduction

The human immunodeficiency virus type-1 (HIV-1) long terminal repeat (LTR) acts as an inducible promoter which can be selectively activated by the *trans*-activator protein, Tat (for review see Jones, 1997). 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.

Activation of transcriptional elongation occurs following the recruitment of Tat to the transcription machinery via a specific interaction with an RNA-regulatory element called the *trans*-activation-responsive region (TAR) (Keen *et al.*, 1997), a 59-residue stem-loop RNA found at the

5' end of all HIV-1 transcripts. Tat recognition of TAR is centred on a U-rich bulge present near the apex of the TAR RNA stem-loop structure (Dingwall *et al.*, 1989, 1990).

After binding to TAR RNA, Tat also interacts with a specific protein kinase called TAK (Tat-associated kinase) (Herrmann and Rice, 1995). Zhu *et al.* (1997) recently cloned the kinase subunit of TAK, CDK9, and discovered that it is analogous to a component of a positive-acting elongation factor isolated from *Drosophila* called pTEFb (Marshall and Price, 1992; Marshall *et al.*, 1996). Direct evidence for the role of TAK in transcriptional regulation of the HIV LTR comes from experiments using inactive mutants of the CDK9 kinase expressed *in trans* to inhibit transcription (Mancebo *et al.*, 1997; Yang *et al.*, 1997; Gold *et al.*, 1998). Furthermore, immunodepletion of HeLa nuclear extracts using antibodies directed against CDK9 demonstrated that the depleted extracts were unable to produce long transcripts from the HIV LTR (Mancebo *et al.*, 1997; Zhu *et al.*, 1997). A critical role for TAK in HIV transcription is also demonstrated by selective inhibition of Tat activity by low molecular weight kinase inhibitors. Tat-dependent transcription from the HIV LTR is strongly inhibited by low concentrations of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) (Marciniak and Sharp, 1991) and a set of related protein kinase inhibitors derived from nucleosides (Mancebo *et al.*, 1997) due to the selective inhibition of CDK9 by these compounds.

A second line of evidence suggesting that the TAK kinase participates directly in the transactivation mechanism is the observation by Wei *et al.* (1998) that the cyclin component of TAK, cyclin T1, participates in TAR RNA recognition. It has been known for several years that mutations in the apical loop region of TAR RNA abolish Tat activity (Feng and Holland, 1988; Rittner *et al.*, 1995), yet this region of TAR is not required for binding by recombinant Tat protein *in vitro*, suggesting that the loop region acts as a binding site for essential cellular co-factors (Dingwall *et al.*, 1989, 1990). Wei *et al.* (1998) have reported that Tat is able to form a ternary complex with TAR RNA and cyclin T1. Formation of the ternary complex requires a functional loop sequence, suggesting that activation of the TAK kinase by Tat requires the participation of the TAR RNA loop.

In addition to cyclin T1, the cyclin component of TAK described by Wei *et al.* (1998), two additional CDK9-associated cyclins, cyclin T2a and cyclin T2b have now been identified (Peng *et al.*, 1998). However, only cyclin T1 is able to associate with Tat and modulate recognition of TAR RNA (Herrmann *et al.*, 1998).

Although most studies of the HIV-1 *trans*-activation mechanism have emphasized 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 a low level of non-processive (basal) transcription in the absence of Tat. Extensive mutagenesis studies of the HIV-1 LTR have failed to identify any DNA elements which 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 (Rittner *et al.*, 1995). The HIV-1 LTR can therefore be considered to be a 'defective' promoter that lacks critical regulatory elements which are required to set up transcription by a fully processive polymerase. Since a number of reports have suggested that cellular enhancer elements may act by promoting the assembly of processive polymerases at promoters (Yankulov *et al.*, 1994; Brown *et al.*, 1998), we decided to test whether a strong cell-specific enhancer element, the immunoglobulin heavy chain enhancer (IgHE) (Banerji *et al.*, 1983; Gillies *et al.*, 1983), could provide the signals necessary to recruit an elongation-competent polymerase to the HIV-1 LTR. The results demonstrate that the IgHE can promote efficient transcription from the HIV LTR using a novel mechanism which does not require TAK.

Results

Activation of transcription from the HIV-1 LTR by the IgHE

The IgHE is a strong tissue-specific enhancer which is active only in cells of the B cell lineage and certain T cell lineages (Banerji *et al.*, 1983; Gillies *et al.*, 1983). To investigate the effects of this element on transcription from the HIV-1 LTR, a 1.0 kb fragment containing the enhancer region was cloned upstream from the start of transcription (into the *EcoRV* site, nt -340). Transcript levels in transiently transfected HeLa cells and B cells were measured by RNase-protection assays using the MJW-10 probe which overlaps the start of transcription in the HIV-1 reporter plasmids. This probe produces a 328 nt protected product corresponding to the HIV-1 mRNAs and a 250 nt protected fragment corresponding to transcripts from the pSV2CAT control plasmid (Figure 1A).

Following transfection of HeLa cells (where the IgHE is inactive), basal (Tat-minus) transcription is low both in the absence and in the presence of the IgHE (Figure 1B). Transcription from both constructs is stimulated <20-fold by pC63-4-1, which expresses Tat under the control of the Moloney murine leukaemia virus LTR (Dingwall *et al.*, 1990). When a similar experiment was performed in a human B cell line (Figure 1C), the basal level of transcription in the presence of the enhancer, was increased 6.7-fold, compared with the basal level of transcription in the absence of the IgHE. Tat-stimulated transcription from the wild-type HIV-1 LTR was 7.9-fold, but produced only a 1.7-fold activation of the LTR containing the IgHE (Figure 1C). In the B cell experiments, Tat was expressed under the control of an SV40 promoter using the plasmid pPL12 since pC63-4-1 was found to express Tat inefficiently in this cell type.

Enhancer-activated transcription is independent of TAR

The TAR element is required both to recruit Tat and to form a complex between Tat and cyclin T1. To test

Fig. 1. The IgHE increases basal transcription from the HIV-1 LTR in B cells. (A) Structure of the plasmids. The pD5-3-3 plasmid carries the HIV-1 LTR and the CAT gene. In pMJW-9, the IgHE was inserted into the *EcoRV* site located 340 nt upstream from the start of transcription in the HIV-1 LTR. The RNA fragments resulting from protection of the HIV-1 reporter (328 nt) and the pSV2CAT control (250 nt) transcripts by the MJW-10 probe are shown. (B) Transcription in HeLa cells. Cells were transfected with 500 ng of pD5-3-3 (-Enhancer) or pMJW-9 (+Enhancer), together with 500 ng of pSV2CAT and between 0 and 100 ng of pC63-4-1 (Tat). The positions of the protected HIV-1 and control mRNAs are indicated on the gel. (C) Transcription in B cells. DG75 cells were electroporated with 15 µg of pD5-3-3 (-Enhancer) or pMJW-9 (+Enhancer), together with 15 µg pSV2CAT and between 0 and 100 ng of the Tat-expressing plasmid pPL12 (Tat).

whether a functional TAR element is required for enhancer-mediated transcription, B cells were transfected using constructs containing mutations either in the Tat-binding site of the TAR element (mGC) (Churcher *et al.*, 1993) or in the apical loop sequence, which is required for cyclin T1 binding to Tat and TAR (mLP) (Wei *et al.*, 1998).

HIV-1 LTRs carrying the mGC and mLG mutations show basal transcription levels which are similar to those seen for the wild-type LTR. As expected, transcription from plasmids carrying these defective TAR elements is not stimulated by Tat (Figure 2).

Addition of the IgHE to the wild-type HIV LTR increased transcription 7-fold. However, further activation of the LTR carrying the IgHE by Tat resulted in only a 1.8-fold increase in RNA synthesis. Transcription from

Fig. 2. Enhancer-activated transcription in B cells is TAR-independent. B cells were electroporated with 15 μ g of reporter plasmids together with 15 μ g pSV₂CAT and 0–50 ng of the Tat-expressing plasmid pPL12. Reporters carrying wild-type TAR sequences were: pD5-3-3 (–Enhancer) or pMJW-9 (+Enhancer). The mGC reporters (pCM78, –Enhancer; pMJW-11, +Enhancer) contain a mutation in the Tat-binding site (G26-C39→C26-G39). The mLG reporters (pC120-3-1-1, –Enhancer; pMJW-12, +Enhancer) contain a mutation in the apical loop sequence (GGG→UUU).

LTRs carrying mutant TAR elements was activated to the same extent as the wild-type LTR by the enhancer, but as expected, there was no further increase in transcription when Tat was expressed *in trans*.

Thus, activation of transcription from the HIV-1 LTR by the enhancer element occurs through a mechanism which is independent of both Tat and TAR RNA.

The NF- κ B sites are not required for enhancer-mediated transcription

The core promoter of the HIV-1 LTR includes three Sp1 sites, the TATA element and a specialized INR element. HIV-1 LTRs carrying mutations in each of these elements can be activated by Tat but the total level of transcription is limited by poor initiation rates (Rittner *et al.*, 1995).

In addition to these core elements, the HIV-1 LTR contains a series of binding sites for cellular transcription factors, including NF- κ B, which could potentially function as enhancer elements and stimulate either transcription initiation or elongation (Nabel and Baltimore, 1987; Perkins *et al.*, 1993). To determine whether any of these sites are required for transcription mediated by the immunoglobulin enhancer, transient transfections were performed using HIV-1 LTRs carrying the SP3 deletion (–340 to –80 nt), which removes all sequences upstream of the core promoter (Figure 3).

In B cells, the SP3 deletion reduced transcript production below detectable levels in the absence of the enhancer (Figure 3). However, when the IgHE was present upstream of the deleted LTR in the SP3 construct, basal transcription was restored to a level equivalent to the Tat-activated level from the wild-type LTR. Tat was unable to further increase transcription from this construct by any significant amount. The immunoglobulin enhancer is therefore able to restore high level transcription from an LTR, which lacks the elements necessary to initiate transcription in its absence.

In HeLa cells, LTRs carrying the SP3 deletion showed an ~7-fold reduction in basal transcription compared with the wild-type. As expected, addition of the enhancer to the SP3 construct did not increase transcription in HeLa cells or inhibit the Tat response (data not shown).

Fig. 3. The IgHE stimulates high level basal transcription from a truncated LTR which lacks NF- κ B sites. B cells were electroporated as described in the legend to Figure 1C. Reporters carried either the wild-type LTR (pD5-3-3, –Enhancer; pMJW-9, +Enhancer) or LTRs containing the SP3 deletion (pMJW-1, –Enhancer; pMJW-5, +Enhancer). Note that in the absence of the enhancer there is no detectable transcription from the reporters carrying the SP3 deletion, but the IgHE is still able to activate basal transcription levels effectively.

Fig. 4. The IgHE stimulates processive transcription from the HIV-1 LTR. (A) Structure of the reporter plasmids and the positions of the proximal (nt –10 to +59) and distal probes (nt +342 to +482) used for RNase-protection analysis. The proximal probe overlaps the start of transcription and provides a measure of initiation, and the distal probe provides a measure of elongation capacity. (B) RNase-protection analysis. B cells were electroporated with 15 μ g of reporter plasmids (pD5-3-3, –Enhancer; pMJW-9, +Enhancer) together with 0–50 ng of the Tat-expressing plasmid pPL12. The positions of the proximal and distal probe protected products are indicated.

The IgHE stimulates highly processive transcription

To determine whether the IgHE stimulates transcription from the HIV-1 LTR by stimulating initiation or elongation, RNase-protection assays were carried out using probes proximal and distal to the promoter (Figure 4A). The proximal probe stretches from nt –10 to +59, and therefore

Fig. 5. Tat-activated transcription, but not enhancer-activated transcription, is selectively inhibited by short-term treatment with high concentrations of DRB. B cells were electroporated with 15 μ g of reporter plasmids (pD5-3-3, -IgHE; pMJW-9, +IgHE) together with 0 (-) or 50 ng (+) of the Tat-expressing plasmid pPL12. Following incubation at 37°C for 46 h, cells were treated with 0, 100 μ M or 200 μ M DRB for 2 h, and the RNA analysed by RNase-protection experiments. (A) RNase-protection analysis of RNA samples was performed as described in the legend to Figure 4 using the proximal (P) and distal (D) probes. Lane 1 contains 32 P-labelled DNA markers, and lanes 2 and 3 show the relative sizes of the undigested proximal and distal probes respectively. The positions of the transcripts protected by the proximal and distal probes are indicated. (B) Quantitative analysis of RNA expression levels in the presence of DRB and in the absence (-E) and presence (+E) of the IgHE. The data is expressed as a percentage of the transcript levels detected by the distal probe in control experiments performed in the absence of DRB.

hybridizes to all transcripts which initiate at the HIV-1 LTR to produce a protected species of 59 nt. The distal probe corresponds to residues +342 to +482 and can only detect transcripts which have extended through into the CAT gene.

As shown in Figure 4B, the levels of proximal transcripts produced from the wild-type LTR in the presence or absence of Tat are very similar, suggesting that initiation is constant. In the absence of Tat, the levels of transcripts detected with the distal probe are very low as transcription is non-processive, but addition of Tat stimulates elongation and increases the levels of transcripts detected using the distal probe 4.6-fold (Figure 4B).

Addition of the immunoglobulin enhancer to the LTR does not increase levels of transcripts detected by the proximal probe. This demonstrates that the enhancer does not stimulate transcription initiation. However, the immunoglobulin enhancer dramatically increases the level of transcripts detected by the distal probe (Figure 4B), demonstrating clearly that the immunoglobulin enhancer is capable of stimulating highly processive transcription from the HIV-1 LTR in the absence of Tat. The inability of the HIV-1 LTR to stimulate processive transcription in the absence of Tat is therefore likely to be the result of the absence of a strong enhancer element in the LTR.

Enhancer-activated transcription elongation does not require TAK

Transcription from the HIV-1 LTR is selectively inhibited by the nucleoside analogue DRB and related protein kinase inhibitors (Marciniak and Sharp, 1991; Marshall *et al.*, 1996; Mancebo *et al.*, 1997). Recent evidence shows that these drugs act by inhibiting CDK9, the kinase subunit of TAK (Zhu *et al.*, 1997).

Two different experimental approaches were used to determine whether enhancer-driven processive transcription was also sensitive to inhibition by DRB. First, B cells were transfected with constructs containing either the wild-type LTR or the enhancer-driven LTR, and the transfected cells were treated 2 days post-transfection with high concentrations of DRB for 2 h prior to analysis of transcript levels (Figure 5A). Analysis of RNA synthesis by RNase-protection experiments using the distal probe showed that Tat-activated transcription was reduced to 57 and 49% of control levels in the presence of 100 and 200 μ M DRB, respectively (Figure 5B). In contrast, enhancer-activated transcription was comparatively insensitive to inhibition by DRB and remained at 82 and 74% of control levels in the presence of the same concentrations of DRB.

In this experiment, we also observed inhibition of the basal level of transcription from the HIV LTR. Mancebo *et al.* (1997) have also observed that basal transcription can be inhibited by protein kinase inhibitors which selectively inhibit TAK activity. This suggests that TAK may be required for transcription from the HIV LTR even in the absence of Tat.

In a second series of experiments, cells were treated for 48 h with low levels of DRB immediately following transfection (Figure 6). Under these conditions, 10 or 20 μ M DRB strongly inhibited Tat-dependent transcriptional elongation, as detected by hybridization to the distal probe (Figure 6A). In contrast, basal transcription and enhancer-dependent transcription levels remained unaffected. As expected, proximal transcript levels, which provide a measure of transcription initiation, remained constant in the presence of 10 or 20 μ M DRB (Figure 6A).

The effect of DRB on transactivation by Tat is also

Fig. 6. Enhancer-activated transcription is unaffected by long-term treatment of cells with low levels of DRB. B cells were electroporated with 15 μ g of reporter plasmids (pD5-3-3, -IgHE; pMJW-9, +IgHE) together with 0 (-) or 50 ng (+) of the Tat-expressing plasmid pPL12 and allowed to recover at 37°C for 2 h. Cells were then treated with 0, 10 or 20 μ M DRB for 48 h. (A) RNase-protection analysis using distal and proximal probes, as described in the legend to Figure 4. The positions of the protected products are indicated. (B) Transactivation by Tat in the absence and presence of DRB. Full-length transcripts were detected by hybridization to the distal probe and the amount of protected transcript measured by Phosphor-Imager analysis. Data obtained using the distal probe to detect transcription were normalized using data from the proximal probe. Transactivation by Tat, or the IgHE, was determined relative to the level of transcripts detected in the absence of Tat and in the absence of DRB (lane 1). (C) Relative RNA expression in the absence (top panel) and presence (bottom panel) of the IgHE. In this graph, transcript levels following DRB treatment are expressed as the percentage of the transcript levels detected in control experiments performed in the absence of DRB.

shown in Figure 6B. In this analysis, the level of transcripts detected by the distal probe was normalized using the data obtained using the proximal probe to provide a measure of transcription initiation (and serve as a control for transfection efficiency). As shown in Figure 6B, transactivation was reduced from 3.7- to 1.5-fold in the presence of 20 μ M DRB. In contrast, the enhancer was able to increase transcription from the LTR in the absence of Tat by 4.6-fold. DRB did not significantly inhibit transcriptional activation by the enhancer. Transcription in the presence of the enhancer increased 3.7- and 3.1-fold in the presence of 10 and 20 μ M DRB, respectively.

As reported above, Tat is able to produce a modest increase in transcription from the enhancer-activated LTR. In the presence of Tat, transcription from LTRs carrying the IgHE was increased 6.2-fold. Addition of DRB selectively inhibited the Tat-dependent component of transcription from the enhancer-activated LTR. Thus, in the presence of 10 and 20 μ M DRB transactivation from the enhancer-activated LTR in the presence of Tat was 4.5- and 4.2-fold respectively. These values are nearly identical to the 4.6-fold increase in transcription resulting from addition of the IgHE to the HIV LTR in the absence of Tat.

Another method for analysing the data quantitatively is to compare the relative levels of RNA expression in the presence and absence of DRB. In the analysis shown in Figure 6C, RNA transcript levels detected by the distal probe were first normalized using the data obtained using the proximal probe, and the transcript levels in control experiments performed in the absence of DRB were considered to represent 100% transcription. The data shows that Tat-activated transcription was reduced to 41% of control levels in the presence of 20 μ M DRB. In contrast, Tat-independent transcription from the wild-type LTR remained at 98% in the presence of 20 μ M DRB. Enhancer-activated transcription in the absence or presence of Tat was also comparatively resistant to DRB treatment and remained at 68% (Figure 6C).

Thus, enhancer-activated transcription is much less sensitive to DRB than Tat-activated transcription. It therefore seems likely that the enhancer activates transcription through a TAK-independent mechanism.

Additional evidence that TAK is required for Tat-dependent transcription, but not for enhancer-dependent transcription, comes from experiments in which CDK9 was expressed *in trans* (Figure 7). Previous reports have

Fig. 7. Inhibition of TAK activity by CDK9 expressed *in trans* reduces Tat-activated, but not enhancer-activated, transcription from the HIV LTR. **B** cells were electroporated as described (in legend to Figure 6) in the presence of 5 μ g of wild-type or D167N (mutant) CMV2-FLAG-CDK9 plasmid or 5 μ g of CMV2-FLAG vector as a control (0). **(A)** RNase-protection analysis was carried out using distal and proximal probes as described in the legend to Figure 4. **(B)** The effect of expression of wild-type CDK9 on distal transcript levels. Full-length transcripts were detected by hybridization to the distal probe and the amount of protected transcript measured by PhosphorImager analysis. Experiments were performed as in (A) using 2 or 5 μ g of wild-type CDK9 DNA. RNA expression levels were determined as a percentage of the transcript levels detected in the absence of added CDK9 and results show the mean of three experiments carried out using 2 μ g of CDK9 DNA and the mean of two experiments carried out using 5 μ g of CDK9 DNA. **(C)** The effect of expression of mutant CDK9 (D167N) on distal transcript levels. Experiments and analysis were carried out as in (B).

shown that transient overexpression of CDK9 specifically inhibits Tat activation of both transfected and integrated HIV LTRs (Mancebo *et al.*, 1997; Gold *et al.*, 1998). A mutation in the catalytic domain of CDK9, D167N, is

more efficient than the wild-type CDK9 in inhibiting Tat-dependent transcription (Gold *et al.*, 1998).

As shown in Figure 7A, expression of the wild-type and mutant CDK9 genes selectively inhibited Tat-dependent transcription. When cells were co-transfected with 5 μ g of either the wild-type CDK9 plasmid or the mutant CDK9 plasmid, Tat-dependent transcription was strongly inhibited. In the presence of the CDK9 plasmids, the level of long transcripts were reduced to basal levels (Figure 7A, compare lane 2 with lanes 6 and 10). In contrast, enhancer-dependent transcription was uninhibited by expression of CDK9 (Figure 7A, compare lane 3 with lanes 7 and 11). As expected, the inhibition of TAK activity by overexpression of CDK9 had no effect on proximal transcript levels (Figure 7A).

Quantitative analysis of the effects of CDK9 expression on Tat-dependent and -independent transcription is shown in Figure 7B and C. In order to compare the effects of CDK9 on the wild-type HIV LTR and the enhancer-driven LTR, the data were normalized to control values measured in the absence of added CDK9 plasmid. Wild-type CDK9 did not efficiently inhibit Tat-dependent transcription at 2 μ g DNA levels, but when 5 μ g of DNA was used, Tat-dependent transcription was reduced to 28% of control levels. Mutant CDK9, however, was able to effectively inhibit Tat-activated transcription at both concentrations of plasmid, reducing distal transcript levels to 37 and 32% of control levels at 2 and 5 μ g, respectively. Enhancer-activated transcription was relatively unaffected by expression of either wild-type or mutant CDK9 and remained at 98 and 61% respectively, in the presence of 5 μ g of either wild-type or mutant.

Thus, enhancer-mediated processive transcription occurs through a distinct mechanism, which does not involve the Tat-associated cofactor, TAK.

Discussion

The HIV-1 LTR is a 'defective' promoter

The 'core promoter' in the HIV LTR contains all the elements required for transcription initiation *in vitro*, including two tandem NF- κ B sites, three tandem Sp1 sites, a TATA element, and an initiator sequence (INR) (Garcia *et al.*, 1989; Liu *et al.*, 1992; Perkins *et al.*, 1993; Rittner *et al.*, 1995). Located upstream of the core promoter elements are binding sites for a variety of cellular transcription factors including TCF-1a, USF, NF-AT, AP-1 and COUP. These upstream elements appear to have a role in regulating viral transcription in a cell type-specific manner and are particularly important in the re-activation of virus from a latent to a lytic stage.

Previous studies have shown that deletion of core promoter elements in the HIV-1 LTR results in reduced transcription initiation levels but does not lead to constitutive activation of transcription (Berkhout *et al.*, 1990; Perkins *et al.*, 1993; Rittner *et al.*, 1995). This suggests that there are no regulatory elements in the LTR that inhibit transcription initiation by elongation-competent polymerases. The HIV-1 LTR can therefore be considered to be a 'defective' promoter which lacks essential elements found in cellular promoters. To test this hypothesis, we introduced the IgHE 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.

RNase-protection experiments have shown that initiation levels in the presence or absence of the IgHE were equivalent and that transcription complexes initiated in the presence of the enhancer were highly processive. Indeed, if the IgHE acted to increase initiation at the LTR rather than elongation, then the effects of the enhancer and Tat would be expected to be synergistic, as was demonstrated in studies of HIV-1 LTR activation using a combination of adenovirus E1a protein, which is known to stimulate initiation, and Tat (Laspia *et al.*, 1990). Instead, we have found that Tat stimulates transcription from the enhancer-activated LTR by <2-fold.

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 cytomegalovirus (CMV) enhancer to the HIV-1 LTR led to an increase in basal transcription and a reduced Tat response. However, both groups only measured total transcription and did not study the effects of enhancers on initiation and elongation rates.

Activation of elongation by Tat and enhancers

Although the immunoglobulin enhancer and Tat both stimulate transcription elongation, they act through distinct mechanisms. There is now general agreement that Tat activation of elongation is mediated by the TAK kinase (Mancebo *et al.*, 1997; Zhu *et al.*, 1997; Gold *et al.*, 1998; Peng *et al.*, 1998). We have recently obtained additional evidence for this hypothesis by demonstrating that stimulation of the CDK9 (PITALRE) kinase by Tat leads to increased phosphorylation of the RNA polymerase II C-terminal domain (CTD) in paused elongation complexes (C. Isel and J. Karn, in preparation). In addition to the CTD, a variety of other proteins in the transcription complex have been reported to be substrates for the CDK9 kinase (Zhu *et al.*, 1997; Zhou *et al.*, 1998).

Because of the central role of TAK in the activation of elongation by Tat, transcription from the HIV LTR is strongly inhibited by the kinase inhibitor DRB which selectively inhibits CDK9 activity (Marciniak and Sharp, 1991; Marshall *et al.*, 1996). Similarly, Tat-activated transcription can be 'squashed' by overexpressed CDK9 (Mancebo *et al.*, 1997; Gold *et al.*, 1998). The experiments reported here show that IgHE-driven transcription is not strongly inhibited by DRB, or by overexpression of CDK9 *in trans*, and therefore, surprisingly, it does not require TAK.

Despite considerable research, the mechanism by which enhancers function remains poorly understood. Although most models for enhancer activity have emphasized their role in transcription initiation, the experiments reported here provide strong evidence that an enhancer element can also stimulate transcription elongation. Consistent with this observation, several studies have shown that stimulation of transcription by VP16 and other factors containing acidic transcription activation domains involves both an increase in initiation and an alteration of RNA polymerase processivity (Yankulov *et al.*, 1994; Krumm *et al.*, 1995; Blair *et al.*, 1996). Although it is not known whether CDK9 plays a role in VP16 activation of transcription, the available genetic evidence suggests that

acidic activators do not require the TAK kinase. For example, Ghosh *et al.* (1993) have shown that Tat is able to synergistically activate a promoter that is driven by VP16 introduced via a fusion protein which binds to DNA.

The simplest explanation for the link between initiation and elongation is that certain transcriptional activators are used to recruit specific elongation factors to the promoter. Likely candidates for this role include CTD kinases that are distinct from TAK. The presence of an intact CTD is required for the efficient activation of transcription by a variety of enhancers, including IgH (Gerber *et al.*, 1995). In addition, the viral *trans*-activator proteins VP16 and E1A have been shown to associate with CDK8 (Gold *et al.*, 1996), a novel CTD kinase which is activated independently of the CDK9 (TAK) and CDK7 (TFIIH) kinases (Herrmann *et al.*, 1996; Gold *et al.*, 1998).

Implications for viral latency

An important feature of the HIV-1 growth cycle is its ability to establish a latent infection in quiescent T cells and thereby escape detection by the immune system (Grossman *et al.*, 1998). In quiescent T cells the proviral genome remains transcriptionally silent until the host cells become activated by antigen activation.

Re-activation of the proviral genome is associated with the induction of functional NF- κ B (Nabel and Baltimore, 1987; Perkins *et al.*, 1993). It is important to note that although the NF- κ B sites in the HIV LTR are often referred to as the HIV-1 'enhancer' elements (Nabel and Baltimore, 1987; Perkins *et al.*, 1993), these sites are actually acting as promoter-proximal activating elements in the HIV LTR. As shown here, NF- κ B elements are required for efficient transcription initiation in HeLa and B cells in the absence of enhancer elements. However, the NF- κ B elements are unable to constitutively activate transcription elongation in the absence of Tat. In contrast, when the IgHE is present, transcription by an elongation-competent polymerase is favoured, and there is a high level of transcription in the absence of Tat. Moreover, the IgHE permits efficient initiation to take place in the absence of the NF- κ B elements.

Our observation that a strong cellular enhancer can permit Tat-independent, highly processive transcription from the HIV LTR, raises the intriguing possibility that the HIV-1 LTR also contains enhancer elements which are transiently activated during T-cell activation. In support of this hypothesis, it has been reported that mitogen activation of HIV-1 transcription can occur in the absence of functional NF- κ B sites (Sakaguchi *et al.*, 1991) and that transcription of the HIV-1 LTR in phorbol ester treated T cells is independent of TAR (Harrich *et al.*, 1990). It will be important to determine whether HIV-1 exploits enhancer-driven transcription to permit a short burst of Tat-independent elongation-competent transcription during proviral activation.

Materials and methods

Plasmids

Test plasmids used were derived from the D5-3-3 plasmid, which contains an LTR and ~300 nt of 5' flanking sequence from the infectious proviral clone HIV_{NL4-3} (Dingwall *et al.*, 1990). Test plasmids carrying TAR elements with the mGC mutation (G26-C39→C·G) in the Tat-

binding site and the mLG mutation in the apical loop of TAR (G3→U3) were derived from the CM78 and C120-3-1-1 plasmids (Dingwall *et al.*, 1990). The murine IgHE was isolated on a 1 kb *Xba*I fragment from plasmid p294 (kind gift of M.Neuberger). The fragment was end-filled using the Klenow fragment of DNA polymerase, and inserted in the sense orientation into the *Eco*RV site 340 nt upstream from the start of transcription (–340) in D5-3-3, CM78 and C120-3-1-1 to give plasmids MJW-9 (wild-type), MJW-11 (mGC) and MJW-12 (mLG), respectively.

The MJW-1 plasmid containing a deletion of the LTR upstream of the Sp1 sites was derived from MTX-3 (SP3) (Rittner *et al.*, 1995). In MJW-1, the synthetic terminator and *gag* gene located between the *Hind*III (+78) and *Bam*HI (+588) sites of MTX-3 was replaced with the CAT gene from D5-3-3 isolated on a *Hind*III (+78) to *Bam*HI (+1710) fragment. The immunoglobulin enhancer was cloned into an end-filled *Xho*I site located at position –80 of MJW-1 to give plasmid MJW-5. MJW-4 is similar to MJW-1 but was derived from MTX-9 and contains a deletion of LTR upstream of the TATA box.

The plasmid MJW-10 contains the *Xho*I (–44) to *Eco*RI (+328) fragment from MJW-4 cloned in the antisense orientation into pBS SK+ (Promega) between the *Eco*RI and *Xho*I sites in the polylinker and was used to prepare probes for the RNase-protection assays. MJW-10 was linearized with *Xho*I and the probe produced was directed against nt –44 to +328 of the HIV reporter plasmid transcripts producing a full-length protected product of 328 nt. The same probe also hybridized to transcripts produced from the pSV2CAT control to give a 250 nt protected product (+78 to +328 nt) which was used as an internal control for transfection efficiency. The plasmid MTX-89 (Rittner *et al.*, 1995) was linearized with *Hind*III to generate an antisense proximal probe (nt –10 to +59). Plasmid MTX-147 carries antisense sequences derived from the CAT gene of D5-3-3 (nt +342 to +482) between the *Hind*III and *Xho*I sites of pBS SK+ and was linearized with *Xho*I to generate an antisense distal probe.

Transfection of cells

HeLa cells (ATCC) were maintained in DMEM (Gibco-BRL) containing penicillin (100 U/ml) and streptomycin (100 µg/ml) supplemented with 10% fetal calf serum (FCS) (Viralex, PAA Laboratories). The Epstein-Barr virus negative Burkitt's lymphoma cell line DG75 was provided by Professor M.Rowe, University of Cardiff, and was maintained in suspension culture in RPMI 1640 media (Gibco-BRL) containing penicillin and streptomycin supplemented with 10% FCS. All cells were cultured at 37°C in 7% CO₂.

HeLa cells were grown to ~80% confluence and were then re-seeded 24 h prior to transfection at a density of 3×10⁵ cells per 10 cm Petri dish. Cells were transfected by the calcium phosphate method using CsCl-purified DNA (Gorman *et al.*, 1983). Each dish received 500 ng of reporter plasmid, 500 ng of control plasmid (pSV2CAT), and 10–100 ng of the Moloney retroviral vector C63-4-1, which carries the Tat gene under the control of the viral LTR (Dingwall *et al.*, 1989). To maintain a constant amount of DNA in each transfection reaction, up to 100 ng of pUC12 DNA was added to compensate for the variable amounts of C63-4-1 DNA present in the samples.

DG75 cells were diluted 1:4 into fresh medium at 24 h before electroporation. Electroporation cuvettes (0.4 cm gap) containing 15 µg of reporter plasmid, 15 µg of control plasmid (pSV2CAT), 0–100 ng of pPL12, a plasmid which expresses Tat under the control of the SV40 promoter, in 50 µl sterile water were pre-chilled for 5 min on ice. pUC12 DNA was used to maintain constant DNA levels in the transfection samples. Cells were resuspended at 2×10⁷ cells/ml in serum-free media and 500 µl of cells added to each cuvette. The cuvettes were chilled on ice for a further 10 min and electroporation carried out at 260 V and 950 µF (BioRad Gene Pulser II). The cuvettes were then incubated at 37°C for 30 min before the dilution of cells into 10 ml of pre-warmed normal growth media.

RNase-protection assays

DG75 cells were harvested by centrifugation 48 h post-transfection, washed in phosphate-buffered saline (PBS), pelleted and snap-frozen on dry ice. Total RNA was extracted using Tri-reagent (Sigma) and treated with 30 U of RNase-free DNase I (10 U/µl, Boehringer Mannheim) for 45 min at 37°C in a total volume of 50 µl containing 1× NEB 2 buffer (New England Biolabs), and 80 units of RNasin (40 U/µl, Promega). RNA was then extracted with phenol/chloroform/isoamyl alcohol and precipitated in ethanol containing 0.5 M ammonium acetate. The DNase I treatment was repeated once and the resulting RNA pellet resuspended in 50 µl of sterile water. Approximately 10 µg of RNA (10–15 µl) was analysed in RNase-protection assays.

HeLa cell monolayers were harvested 48 h post-transfection using a cell lifter following removal of media and washing in PBS. Cells were then pelleted and snap-frozen on dry ice. RNA was extracted as above but was treated once only with 4 units of RNase-free DNase I (2 U/µl, HT Biotechnology Ltd) before RNase-protection analysis.

Antisense probes were prepared using T3 RNA polymerase (Boehringer Mannheim). Transcription reactions (25 µl) contained 1 µg of linearized DNA, 1× transcription buffer (Boehringer Mannheim), 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 µM UTP, 50 µCi [α -³²P]UTP (800 Ci/mmol), 40 U RNasin, and 20 U T3 RNA polymerase (20 U/µl, Boehringer Mannheim). The labelled transcripts produced were purified by urea polyacrylamide gel electrophoresis.

Hybridization reactions (20 µl) containing ~10 µg of total cellular RNA and 100 000 c.p.m. of probe in hybridization buffer (80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA) were heated to 90°C for 3 min to denature the RNA and then incubated at 37°C for 18 h. The reaction mixtures were diluted by the addition of 300 µl of digestion buffer (300 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA) and the single-stranded sequences digested with RNase T₁ (15 µg/ml) and RNase A (1 ng/ml) for 1 h at 30°C. Following extraction with phenol/chloroform/isoamylalcohol the protected RNA fragments were analysed by electrophoresis through 6% urea polyacrylamide gels.

Treatment of cells with DRB

DG75 cells were treated with DRB using two different methods. In the first method, cells were either first electroporated as described above, transferred to 10 ml of growth media and incubated at 37°C for 46 h prior to the addition of DRB to a final concentration of 100 or 200 µM. Following a further 2 h incubation at 37°C, cells were harvested for RNA analysis. In an alternative protocol, cells were first electroporated, and then allowed to recover for 30 min at 37°C before being transferred to 10 ml pre-warmed media containing 10 or 20 µM DRB and incubated for a further 48 h. The DRB was diluted from a 100 or 10 mM stock in dimethylsulphoxide (DMSO), and therefore the final medium contained 0.2% DMSO. Control cells were mock-treated by the addition of DMSO to the media.

Inhibition of TAK-dependent transcription by CDK9 expression

DG75 cells were electroporated as described above using 15 µg of reporter plasmid, 0 or 50 ng of pPL12, and 2 or 5 µg of wild-type FLAG-PITALRE (CDK9) or mutant (D167N) expression plasmids (Gold *et al.*, 1998). Control samples received 5 µg of the vector DNA (CMV2-FLAG, Kodak). DNA levels in each of the transfected samples were adjusted to 5 µg of CDK9 plasmid and vector DNA.

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