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Poly(A) signals and transcriptional pause sites combine to prevent interference between RNA polymerase II promoters

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Transcriptional termination by RNA polymerase II at the 3′ end of genes encoding poly(A)$^+$ mRNAs is thought to require two distinct *cis*-active elements: a functional poly(A) signal and a downstream transcriptional pause site. An important requirement for efficient termination is to prevent transcriptional interference of downstream-located promoters. We have therefore investigated whether these two elements, individually or in combination, can prevent transcriptional interference of RNA polymerase II-activated promoters. For this purpose, we constructed an expression plasmid containing two tandem retroviral long terminal repeats (LTRs) derived from HIV-1. When transfected into HeLa cells, this construct resulted in transcriptional interference of the LTR promoters. Using this assay, we were able to show that a single poly(A) signal was able to protect an otherwise occluded promoter. This effect depended on the RNA-processing strength of the poly(A) signal. Furthermore, transcriptional pause sites provided adequate protection against promoter occlusion even when tested alone. Finally, a combined element consisting of a poly(A) signal followed by a pause site was more efficient in promoter protection than either element on its own. These results indicate that an interference-blocking element can take various forms: a poly(A) signal, a transcriptional pause site or a combination of both.

Key words: interference/termination/transcription

Introduction

One consequence of the tandem arrangement of genes in the genome is that transcription of one gene may downregulate the expression of another gene located downstream with the same 5′ to 3′ orientation. This inhibition is called transcriptional interference or promoter occlusion. Its precise mechanism is unknown, but it is assumed that polymerase complexes initiating at the upstream promoter and reading into the downstream promoter preclude the assembly of functional (pre)initiation complexes at the downstream promoter. Transcriptional interference was first described in *Escherichia coli* (Hausler and Somerville, 1979; Adhya and Gottesman, 1982). Prokaryotes are indeed particularly susceptible to transcriptional interference because of their densely packed genome and this may be one of the reasons for the tight control of transcriptional termination in prokaryotes [for a review, see Platt (1986)].

In eukaryotes, transcriptional interference may at first glance seem to be less relevant as their genes are more dispersed throughout the genome. However, interference in eukaryotes is well documented for genes transcribed by RNA polymerase I and II (pol I and II). Pol I-transcribed rRNA genes are arranged in tandem as 'head to tail' expression units and are very actively expressed, which predisposes them to transcriptional interference [for a review on rRNA genes, see Sollner-Webb and Mougey (1991)]. The rRNA promoter is sensitive to transcriptional interference, but it is protected in its natural context by a transcription terminator site situated upstream of each promoter (Bateman and Paule, 1988; Henderson *et al.*, 1989; McStay and Reeder, 1990). Furthermore, *in vitro* studies have shed some light on the molecular mechanism of pol I interference. A specific transcription factor required for pol I initiation is no longer able to stably interact with its binding site when the rRNA promoter is occluded by upstream transcription (Bateman and Paule, 1988; Henderson *et al.*, 1989).

Interference of pol II promoters was first described in retroviruses, notably the avian leukosis virus (ALV), where initiation at the 3′ long terminal repeat (LTR) is repressed by the transcriptionally active 5′ LTR (Cullen *et al.*, 1984). Furthermore, two closely spaced α-globin genes in an artificial gene construct were also shown to interfere with each other (Proudfoot, 1986). Other examples of pol II interference include: (i) the *Drosophila* alcohol dehydrogenase (*adh*) gene where the distal adult promoter downregulates the proximal larval promoter during the adult stage (Corbin and Maniatis, 1989); (ii) the adenovirus E1B-polypeptide IX transcription unit where transcription from the upstream-located E1B promoter prevents initiation from the downstream polypeptide IX promoter during early virus infection (Vales and Darnell, 1989); (iii) the *Saccharomyces cerevisiae* actin gene where a cryptic promoter in the intron is occluded by transcription from the actin promoter at the 5′ end of the gene (Irniger *et al.*, 1992).

Transcriptional interference of pol II promoters can, therefore, occur between tandem promoters transcribing the same transcription unit, between tandem promoters transcribing nested genes or between closely spaced genes. The presence of tandem promoters or closely spaced genes, and hence the possibility of transcriptional interference, may be more widespread. First, the tissue-, development- or stimulus-specific expression of several genes is often mediated by the presence of multiple promoters (Schibler and Sierra, 1987): e.g. the human and murine c-*myc* gene [see Marcu *et al.* (1992) for a review on c-*myc*], the human aldolase A gene (Maire *et al.*, 1987) and the porphybilinogen deaminase gene (Chretien *et al.*, 1988). Second, genes are not evenly spread over the genome and in some cases are positioned close together in clusters. A striking example of this is the major histocompatibility complex (MHC) class III locus which contains at least 36

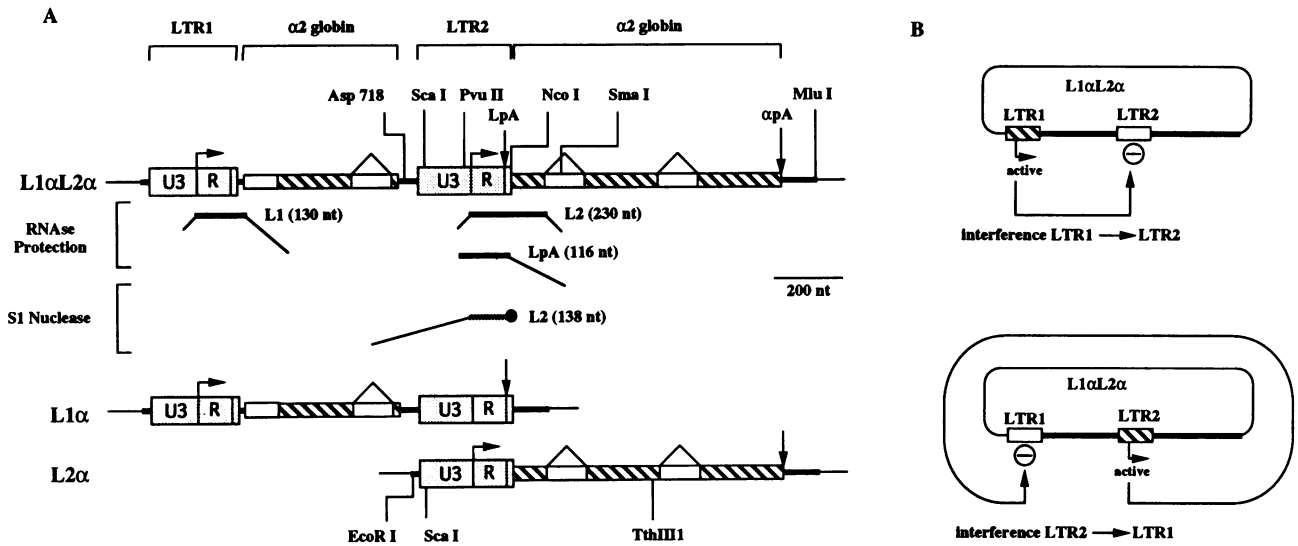

Fig. 1. Schematic drawing of the L1αL2α, L1α and L2α constructs. (**A**) The interference construct L1αL2α contains two nested transcription units L1α and L2α. It consists of an upstream HIV-1 LTR (LTR1), human α2-globin gene sequences (from the *Sma*I site in intron 1 to the *Bst*EII in exon 3), a downstream HIV-1 LTR and human α2-globin gene sequences [from the *Nco*I site in exon 1 to a polylinker *Xba*I site 3′ of the α2 poly(A) site]. Transcripts initiated at LTR1 are polyadenylated at the HIV poly(A) site (LpA) in LTR2. Transcripts initiated at LTR2 are polyadenylated at the α2 poly(A) site (αpA). The L1αL2α plasmid was then further modified as described in the text. In general, poly(A) sites (SPA and αpA) were inserted in the *Asp*718 site and transcriptional pause sites (C2 and α pause) in the *Sca*I site. The L1α-SPA C2-L2α and the L1α-αpA C2-L2α plasmids contain a poly(A) site in the *Asp*718 site and the C2 pause site in the *Sca*I site. A fragment containing the SPA followed by the C2 pause site was also cloned in the *Mlu*I site 3′ of the second transcription unit. Transcriptional activity of LTR1 and LTR2 can be analysed simultaneously in an RNase protection assay using a single antisense RNA probe complementary to the *Pvu*II–*Sma*I region of L2α. Fragments protected upon analysis of cytosolic RNA are depicted below the L1αL2α plasmid by thick horizontal lines with oblique parts corresponding to digested regions. L1: a 130 nt fragment corresponding to the 5′ end of transcripts initiated at LTR1; L2: a 230 nt fragment corresponding to the 5′ end of transcripts initiated at LTR2; LpA: a 116 nt fragment corresponding to the 3′ end of transcripts processed at the poly(A) site (LpA) in LTR2. LTR2 expression was also analysed by means of an S1 nuclease assay using a probe 5′ end-labelled at the *Nco*I site in exon 1 of α2-globin. S1 analysis with this probe yields a 138 nt fragment (L2) corresponding to the 5′ end of transcripts initiated at LTR2. L1α and L2α are expression plasmids which contain only one transcription unit. The L2α unit was further modified by cloning fragments in the *Eco*RI (SPA), the *Sca*I (C2 or α pause) or the *Tth*III1 site (SPA, C2 or α pause). L1αL2α, L1α, L2α and their derivatives were all contained within the pSVod expression vector. (**B**) Interference assay. Transfection of the L1αL2α plasmid into HeLa cells results in mutual interference between LTR1 and LTR2: (i) an active LTR1 downregulates the downstream-located LTR2; (ii) pol II complexes initiated at LTR2 transcribe around the circular plasmid and in doing so interfere with LTR1 (see the text and Figure 2). The ability of poly(A) signals and pause sites to prevent interference was tested by inserting them between LTR1 and LTR2, and examining the effect on LTR2 activity.

genes in 680 kb space and where several genes with the same 5′ to 3′ orientation are separated by only a few hundred nucleotides between the poly(A) site of the upstream gene and the cap site of the next gene [for a review of the MHC class III locus, see Milner and Campbell (1992)]. In such clusters, transcriptional interference may compromise the independent expression of similarly orientated flanking genes, unless there is efficient transcriptional termination in the intergenic region.

One way to prevent occlusion of a downstream-located promoter or gene is through direct transcriptional termination, as is clearly illustrated for the rRNA promoter. However, pol II termination, whether intragenic (also known as attenuation) or at the 3′ end of the transcription unit, is still relatively poorly understood [for reviews, see Proudfoot and Whitelaw (1988), Spencer and Groudine (1990) and Kerppola and Kane (1991)]. For the termination event at the 3′ end of genes coding for poly(A)$^+$ mRNAs, it is now well established that a functional poly(A) signal is an essential component of the pol II termination signal (Whitelaw and Proudfoot, 1986; Logan *et al.*, 1987; Connelly and Manley, 1988; Lanoix and Acheson, 1988). More recently, sequences located downstream of the poly(A) site have also been implicated in the termination process by virtue of their capacity to pause or retard the transcription complex. These observations have led to the hypothesis that the pol II termination signal is actually bipartite, consisting of a poly(A) signal and a downstream pause site (Connelly and Manley, 1989a; Ashfield *et al.*, 1991; Enriquez-Harris *et al.*, 1991; Vandenbergh *et al.*, 1991). The actual mechanism whereby poly(A) signals and pause sites induce termination, is as yet unresolved. One possibility is that a poly(A) signal causes the release of anti-termination factors associated with the transcription complex, thereby rendering it prone to termination (Logan *et al.*, 1987). Alternatively, since the poly(A) signal is essential for termination, it has been proposed that 3′ end cleavage generates an unprotected downstream product. This is then degraded by 5′ to 3′ exonucleases and/or DNA/RNA helicases, thereby destabilizing the transcription complex (Connelly and Manley, 1988; Proudfoot, 1989). In either model, pause sites will increase the likelihood of termination by stalling or slowing down the transcription complex.

In view of the proposed role of poly(A) signals and pause sites in pol II termination, we have investigated how these elements affect interference between pol II promoters. For this purpose, we have devised an interference assay based on two tandem promoters. We provide evidence that each element on its own is capable of protecting an otherwise occluded promoter. Moreover, for poly(A) signals there is a correlation between poly(A) site strength and its protective effect, with a more potent poly(A) site being more active

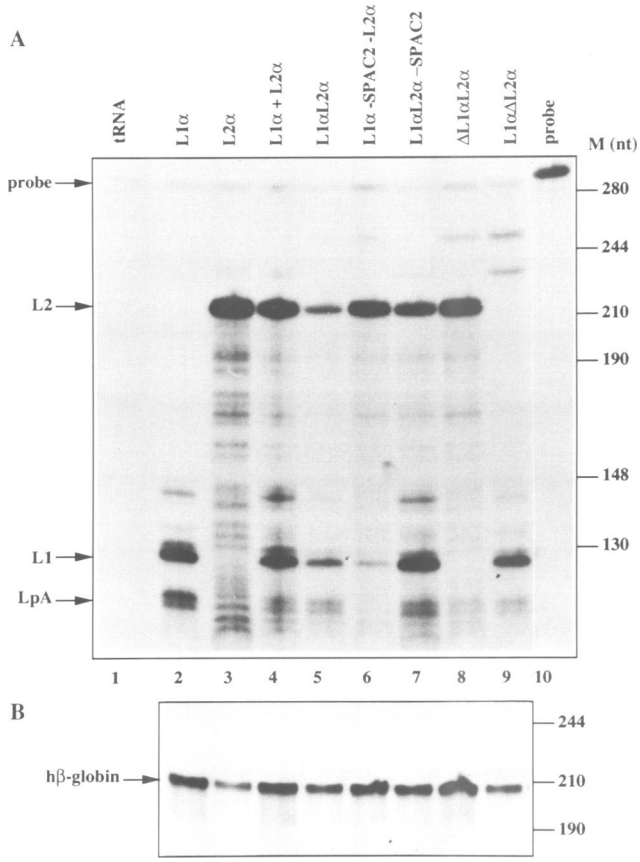

Fig. 2. Interference assay. (**A**) RNase protection assay. HeLa cells were transfected with 4 μg of the plasmids indicated above lanes 2–9. Cytosolic RNA was prepared and analysed by means of an RNase protection assay. The protected fragments L1, L2 and LpA are described in Figure 1. The L1 and L2 signals correspond to transcripts initiated at LTR1 and LTR2, respectively, and they reflect the transcriptional activity of each promoter. Lane 1: RNA probe hybridized to tRNA; lane 2: L1α; lane 3: L2α; lane 4: L1α and L2α co-transfected (4 μg each); lane 5: L1αL2α; lane 6: L1α-SPA C2-L2α; lane 7: L1αL2α-SPA C2; lane 8: ΔL1αL2α; lane 9: L1αΔL2α; lane 10: aliquot of undigested riboprobe. Note that in the interference construct L1αL2α (lane 5), both L1 and L2 signals decrease in comparison with the L1 and L2 signals obtained on transfection in *trans* (lane 4), but that they can be restored to the original level either by deleting the other promoter (lanes 8 and 9) or by insertion of SPA C2, a pol II terminator (lanes 6 and 7). (**B**) S1 nuclease assay. To control for transfection efficiency, the plasmids described in (A) were co-transfected with a human β-globin expression plasmid. Human β-globin mRNA levels were analysed by means of an S1 nuclease protection assay using a 3′ end-labelled probe.

in this assay. Finally, the most efficient protection is observed with a combined signal consisting of a poly(A) signal followed by a pause site.

Results

Experimental outline

To study the effect of pol II termination signals on transcriptional interference, we constructed an expression plasmid (L1αL2α; see Figure 1) containing two HIV-1 LTRs in tandem, each driving the expression of a truncated human α2 globin gene. The HIV-1 LTR fragments used (−138 to +127 in LTR1; −158 to +127 in LTR2 with the numbers relative to the transcriptional start site) consist of the distal part of U3, the entire R region and the proximal part of U5. They therefore contain all the sequences required for efficient expression in HeLa cells, including the NF-κB and the Sp1 sites, the TATA box and the TAR stem loop (Garcia *et al.*, 1987; Greene, 1990; for a review on the HIV promoter). This plasmid was then transfected into HeLa cells together with a Tat expression plasmid (Adams *et al.*, 1988) for transactivation of the HIV-1 LTR and a human β-globin expression plasmid (Proudfoot *et al.*, 1992) to control for transfection efficiency. LTR1 and LTR2 activity was assessed by an RNase protection assay on cytosolic RNA using a single antisense RNA probe that allowed the simultaneous detection of the 5′ ends of LTR1- and LTR2-initiated transcripts (see Figure 1). Alternatively, LTR2 expression was analysed by means of an S1 nuclease assay using a 5′ end-labelled probe that only recognized the 5′ end of LTR2 mRNAs (see Figure 1). The S1 nuclease assays also contained a 3′ end-labelled probe for the co-transfected human β-globin gene, enabling us to normalize the L2 signal relative to the co-transfection control.

LTR1 and LTR2 mutually interfere in the L1αL2α plasmid

We first checked whether transcriptional interference occurred in the parental L1αL2α plasmid. As a genuine interference effect should only be observed in *cis* (when both LTRs are on the same plasmid) and not in *trans*, we compared the activity of the LTR1 and LTR2 promoters in the L1αL2α plasmid with their activity when present on separate plasmids containing only one expression unit (L1α or L2α; see Figure 1). The L1α or L2α plasmids (4 μg each) were transfected into HeLa cells and cytosolic mRNAs were analysed by means of an RNase protection assay. Only minor changes in the respective 5′ end signals were observed between the individual or the combined transfections (Figure 2: lanes 2–4; L1 and L2 refer to the protected fragments of mRNAs initiated as LTR1 or LTR2, respectively). This indicates that there is no significant interaction in *trans* between the two expression units. Qualitatively similar results were obtained when the transfection was done with 1, 4 or 10 μg of plasmids, showing that the level of *trans*-acting factors required for expression was not limiting within this range (data not shown; subsequent experiments were all done with 4 μg of plasmid per transfection). In contrast, when the L1α and the L2α expression units were present in *cis* on the same plasmid as in L1αL2α, both the L1 and the L2 signal decreased as compared with the L1α + L2α transfection, indicating that LTR1 and LTR2 mutually interact in *cis*, but not in *trans* (Figure 2: lanes 4 and 5). That this decrease in 5′ end signal is due to transcriptional interference is corroborated by two additional observations. First, when either LTR1 or LTR2 is deleted (ΔL1αL2α or L1αΔL2α), the 5′ signal of the other promoter (LTR2 or LTR1) is upregulated (Figure 2: lanes 8 and 9). Second, expression of LTR1 or LTR2 could also be rescued by cloning a pol II termination signal downstream of the LTR2 or LTR1 transcription unit (Figure 2: lanes 6 and 7). This termination signal consists of a synthetic poly(A) site (SPA) followed by a transcriptional pause site derived from the C2 downstream region (both elements are described in greater detail in the next sections). As the decrease in LTR1 or LTR2 activity is only observed when the two units are placed in *cis* and not in *trans*, and as LTR1 or LTR2 expression can be restored either by deleting the other

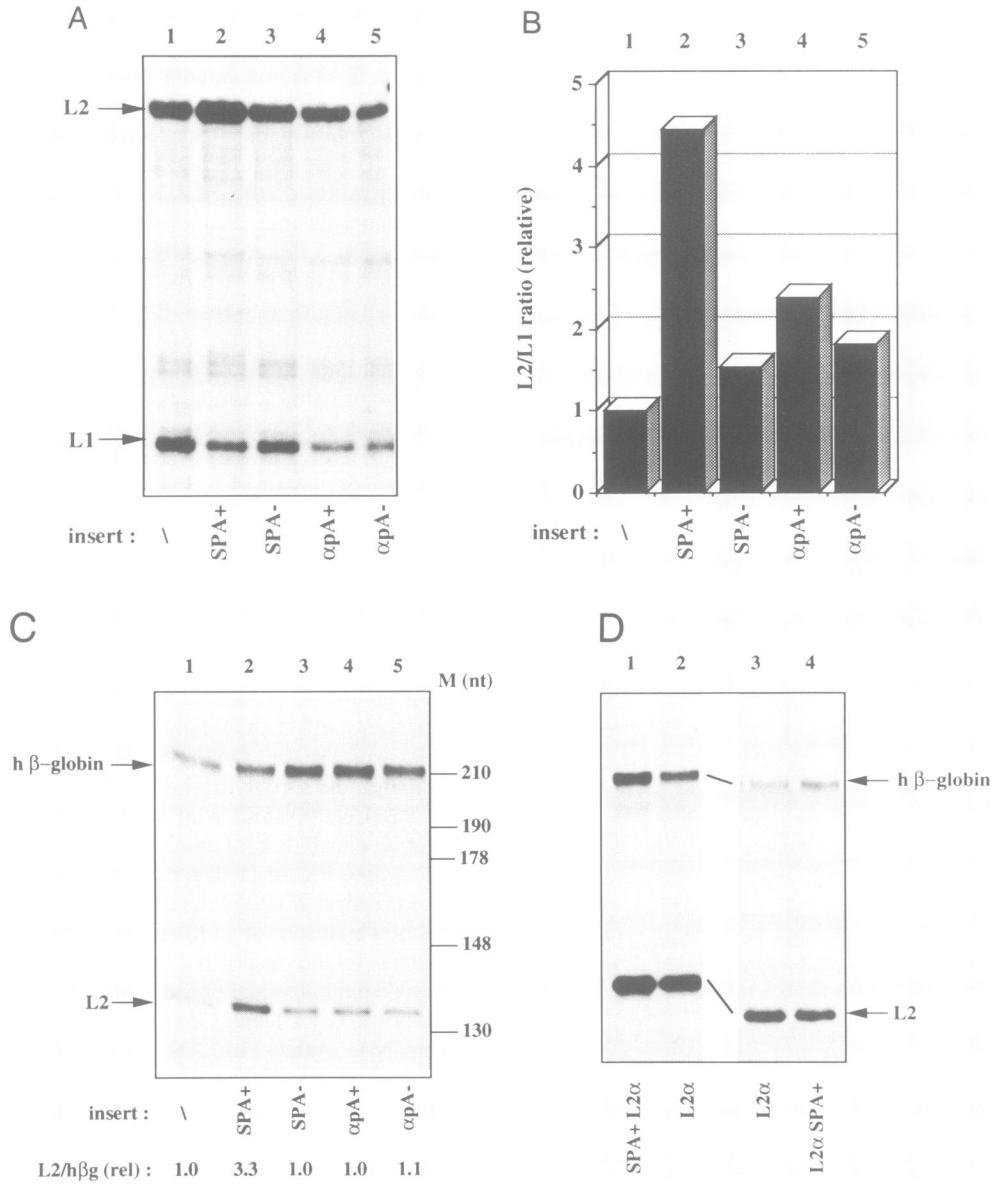

Fig. 3. Poly(A) sites alleviate transcriptional interference depending on their processing strength. (**A**) RNase protection assay. HeLa cells were transfected with the parental L1αL2α plasmid (lane 1) or derivatives containing poly(A) sites in the *Asp*718 site between LTR1 and LTR2 (see Figure 1). Cytosolic RNA was isolated and analysed as in Figure 2A. Lane 1: parental L1αL2α plasmid; lanes 2 and 3: constructs containing the SPA site in sense (lane 2) or antisense orientation (lane 3); lanes 4 and 5: α2 poly(A) site constructs in sense (lane 4) and antisense orientation (lane 5). The L1 and L2 signals correspond to transcripts initiated at LTR1 and LTR2, respectively. The data shown are representative of three independent experiments. (**B**) The autoradiograph in (**A**) was scanned with a laser densitometer to quantify the L1 and L2 signals, and to calculate the ratio of L2 to L1. The ratios were then normalized with the L2/L1 value of L1αL2α taken as 1. An increase in L2/L1 reflects relief of transcriptional interference. SPA in the sense, but not in the antisense orientation, causes a pronounced increase in the L2/L1 ratio. The αpA hardly affects the L2/L1 ratio. (**C**) S1 nuclease analysis. HeLa cells were transfected with the parental L1αL2α plasmid (lane 1) or derivatives containing poly(A) sites in the *Asp*718 site (lanes 2–5) as in panel 3A, together with a human β-globin co-transfection control. Cytosolic RNA was prepared and analysed by means of an S1 nuclease assay using a 5′ end-labelled probe to detect L2 transcripts (see Figure 1) and a 3′ end-labelled probe for the human β-globin co-transfection control. An increase of the L2 signal relative to the co-transfection control, as in lane 2 for SPA+, indicates a relief of transcriptional interference. The S1 data were quantified by laser densitometry to calculate the fold increase in L2 level relative to the co-transfection control. The corresponding values [L2/hβg (rel)] are given below each lane. (**D**) Control S1 nuclease analysis. Plasmids containing the single transcription unit L2α (lanes 2 and 3) and L2α plasmids containing the SPA in sense orientation either upstream (SPA+ L2α: lane 1) or downstream (L2α SPA+: lane 4) of the L2 initiation site were transfected in HeLa cells together with a β-globin expression plasmid. Cytosolic RNA was analysed by means of an S1 nuclease assay using a 5′ end-labelled probe to detect L2 transcripts and a 3′ end-labelled probe for the co-transfection control. Lanes 1–2 and 3–4 are taken from a different experiment. As shown, SPA does not affect the L2 levels in either position.

promoter or by insertion of a transcriptional termination signal, we conclude that LTR1 and LTR2 mutually interfere: not only will an active LTR1 downregulate LTR2, but also transcription initiated on LTR2 will interfere with LTR1, most likely by transcribing around the circular plasmid (see Figure 1B). Evidence for interference around a circular plasmid was also observed in a duplicated α-globin construct (Proudfoot, 1986).

Effectively, a dual effect is observed when the terminator signal is cloned between LTR1 and LTR2 (L1α-SPA C2-L2α; lane 6 in Figure 2): (i) an increase in L2, which reflects the increased transcriptional activity of LTR2 because

it is now protected from interference; (ii) a decrease in the L1 signal because the activated LTR2 now interferes more strongly with LTR1. Both effects combine to increase the ratio of LTR2 to LTR1 transcripts (L2/L1 ratio). We will therefore use this ratio to evaluate the ability of poly(A) signals and pause sites inserted between LTR1 and LTR2 to relieve transcriptional interference. Such a ratio change is less clear in the complementary clone L1αL2α-SPA C2 where the pol II terminator is cloned downstream of LTR2 (lane 7 in Figure 2). In this construct, LTR2 no longer interferes with LTR1 (hence an increase in the L1 signal), but the L2 signal does not decrease significantly. This is due to the fact that the LTR2 transcripts are now processed at a much stronger poly(A) site [SPA versus α2-globin poly(A) site] which will augment the cytosolic mRNA levels (Gil and Proudfoot, 1987). Any decrease in L2 because of stronger interference is therefore neutralized by the stimulatory effect of the stronger poly(A) signal.

Poly(A) signals prevent promoter occlusion

As poly(A) signals are known components of the pol II termination signal (see Introduction), it might be expected that they counteract interference. In the ALV-LTR system, interference was blocked by inserting the SV40 late poly(A) site in the proviral genome (Cullen *et al.*, 1984). However, this does not seem to be a universal property of all poly(A) signals as interference was still observed in an α-globin tandem gene construct in spite of the presence of a functional poly(A) site (Proudfoot, 1986).

To investigate the possible involvement of poly(A) signals in preventing promoter occlusion, we tested two poly(A) sites which are known to differ in their strength as RNA processing signals: (i) the SPA is derived from an oligonucleotide encoding the necessary elements for efficient 3′ end processing [AAUAAA followed by an extensive downstream GU/U sequence (Levitt *et al.*, 1989)]; (ii) the poly(A) site of the human α2 globin gene (αpA) which has a rather limited downstream GU/U element. The relative strength of these two signals has previously been characterized *in vivo* by means of a poly(A) site competition assay. When both sites are placed in tandem, the SPA is nearly exclusively used, indicating that SPA is much stronger than αpA (Ashfield *et al.*, 1991). This is consistent with previous observations that the downstream GU/U sequence contributes to poly(A) site strength (Gil and Proudfoot, 1987).

Both poly(A) signals were cloned into the *Asp*718 site between LTR1 and LTR2 to see whether they could protect the downstream LTR2 promoter. Cytosolic RNA of transfected HeLa cells was first analysed by means of an RNase protection assay (Figure 3A) and the data obtained were quantified by laser densitometry to calculate the ratio of the L2 signal to the L1 signal (Figure 3B). As described in the previous section, this ratio is used as an indicator for interference of the LTR2 promoter. This analysis revealed that SPA was much more efficient in preventing interference of the LTR2 promoter than the αpA. Indeed SPA in the sense, but not in the antisense orientation, caused an ~4-fold increase in the L2/L1 ratio. The orientation dependency of the SPA effect indicates that the SPA is working as a genuine RNA processing signal. In contrast, the weaker αpA had a small effect on the L2/L1 ratio and only part of it could be ascribed to an RNA processing event as the αpA effect was only partially orientation specific (compare αpA+ and αpA− in Figure 3A and B). The RNase protection assay provides information on how LTR2 expression changes with respect to LTR1, but in the absence of an internal co-transfection control it cannot be used to measure changes in the overall levels of L2. It was therefore complemented by means of an S1 nuclease assay in which the L2 signals were analysed with a 5′ end-labelled probe and compared with the expression levels of a human β-globin co-transfection control. The S1 data were quantified by laser densitometry and the fold increase in L2 level relative to the co-transfection control was calculated. The results of such an experiment are shown in Figure 3C. Consistent with the RNase protection assay, SPA in the sense, but not in the antisense orientation, increased the L2 signal, indicating that it protected the LTR2 promoter. Similarly, the αpA site (sense or antisense) did not significantly affect the L2 signal when compared with the parental L1αL2α plasmid. Both assays therefore yield qualitatively identical results. However, the absolute figures for the L2/L1 ratios as calculated from the RNase protection data tend to be higher than the figures for the increase in L2 as derived from the S1 data (see also the next two sections). This most likely reflects the fact that the L2/L1 ratio takes two variables into account, whereas the L2 levels as obtained in the S1 analysis are obtained from only one variable.

It is formally possible that SPA(+) affected the L1 and L2 signals, not because of an anti-interference effect, but because it destabilized the LTR1 transcripts and/or because SPA acted in an enhancer-like mode to stimulate LTR2 activity. To exclude the latter two possibilities, we performed the following control experiment. The single transcription unit L2α was modified by cloning SPA in the *Eco*RI site upstream of the transcription start site in the LTR to give plasmid SPA+ L2α (see Figure 1). Another plasmid, L2α SPA+, was obtained by inserting the SPA downstream of the LTR cap site (see Figure 1). The L2α, SPA+ L2α and L2α SPA+ plasmids were then transfected in HeLa cells together with the β-globin control plasmid. Transcriptional activity of LTR2 was assessed via an S1 nuclease assay and scored against the β-globin control. As shown in Figure 3D, SPA did not affect the L2 signal in either position, indicating that the decrease in L1 observed in the dual transcription unit was not due to a destabilizing effect of SPA nor that the increase in L2 was caused by an enhancer effect of SPA. In view of our observation that two distinct promoters can interfere around a circular plasmid (see Figure 2), a protective effect might have been expected by placing SPA+ upstream of LTR2 in the single transcription unit. This, however, was not observed suggesting that transcriptional interference only occurs with two different promoters on the plasmid and not when there is only one promoter present in the plasmid. We do not have a mechanistical explanation for this observation, but this should not detract from the interpretation of our results as our interference assay is based on a plasmid with two promoters, where we reliably observe transcriptional interference.

We therefore conclude that poly(A) signals are able to block transcriptional interference. However, the degree of this protective effect depends on the processing strength of the poly(A) signal. This may explain why the SV40 late poly(A) site was able to block interference in the ALV-LTR system. The SV40 late poly(A) site is known to be a strong processing site due to the presence of activating sequences

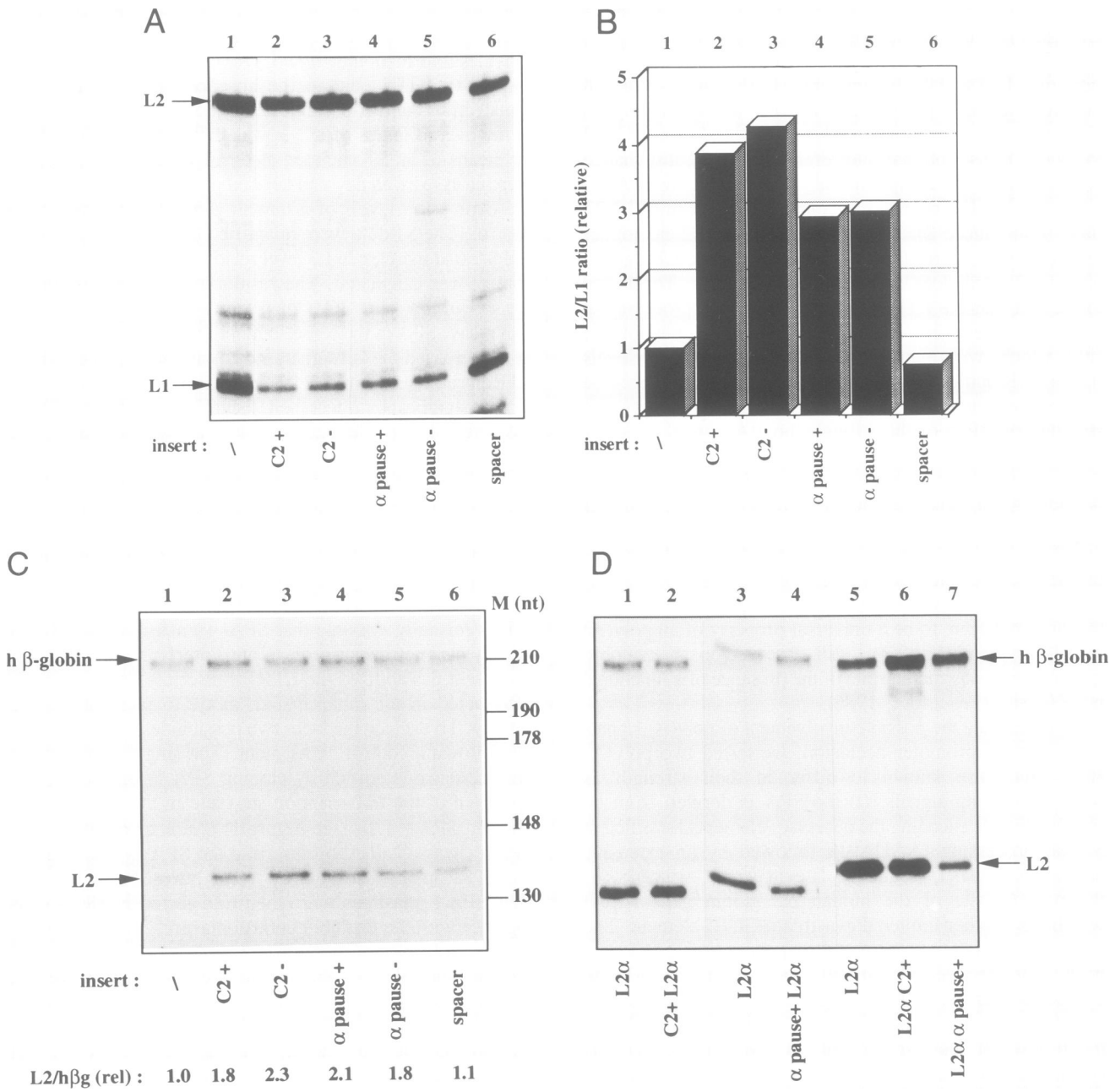

Fig. 4. Transcriptional pause sites prevent interference. (**A**) RNase protection assay. HeLa cells were transfected with the parental L1αL2α plasmid and derivatives containing transcriptional pause sites in the *Sca*I site (see Figure 1). Cytosolic RNA was prepared and analysed as in Figure 2A. Lane 1: parental L1αL2α plasmid; lanes 2 and 3: constructs containing the C2 pause site in the sense (C2+) and antisense orientation (C2−), respectively; lanes 4 and 5: constructs containing the α pause site in the sense (+) and antisense orientation (−), respectively; lane 6: L1αL2α plasmid containing a pGEM7-derived spacer fragment of 105 nt in the *Sca*I site. The data shown are representative of three independent experiments. (**B**) The autoradiograph in (A) was scanned with a laser densitometer to quantify the L1 and L2 signals, and to calculate the ratio of L2 to L1. The ratios were then normalized with the L2/L1 value of the L1αL2α taken as 1. (**C**) S1 nuclease analysis. HeLa cells were transfected with the parental L1αL2α plasmid (lane 1) or derivatives containing pause sites in the *Sca*I site (lanes 2−5) or a neutral spacer fragment (lane 6) as in panel 4A together with a human β-globin co-transfection control. Cytosolic RNA was prepared and analysed by means of an S1 nuclease assay using a 5′ end-labelled probe to detect L2 transcripts and a 3′ end-labelled probe for the co-transfection control. The S1 data were quantified by laser densitometry to calculate the fold increase in L2 level relative to the co-transfection control. The corresponding values [L2/hβg (rel)] are given below each lane. (**D**) Control S1 nuclease analysis. Plasmids containing the single transcription unit L2α and L2α plasmids containing the C2 and α pause sites (sense orientation only) either upstream (C2+ L2α and α pause+ L2α) or downstream (L2α C2+ and L2α α pause+) of the L2 initiation site were transfected in HeLa cells together with a β-globin expression plasmid. Cytosolic RNA was analysed by means of an S1 nuclease assay using a 5′ end-labelled probe to detect L2 transcripts and a 3′ end-labelled probe for the co-transfection control. Lane 1: L2α plasmid; lane 2: C2+ L2α; lane 3: L2α; lane 4: α pause+ L2α; lane 5: L2α; lane 6: L2α C2+; lane 7: L2α α pause+.

upstream and downstream of the cleavage site (Conway and Wickens, 1985; Sadofsky *et al.*, 1985; Carswell and Alwine, 1989). Similarly, the α-globin tandem gene construct contained the weak αpA sites which we have shown to be inefficient in promoter protection.

Effect of transcriptional pause sites on interference

Two transcriptional pause sites have recently been implicated in pol II termination. The α pause site is located some 300 nucleotides downstream of the poly(A) site of the human α2-globin gene. Nuclear run-off experiments, as well as

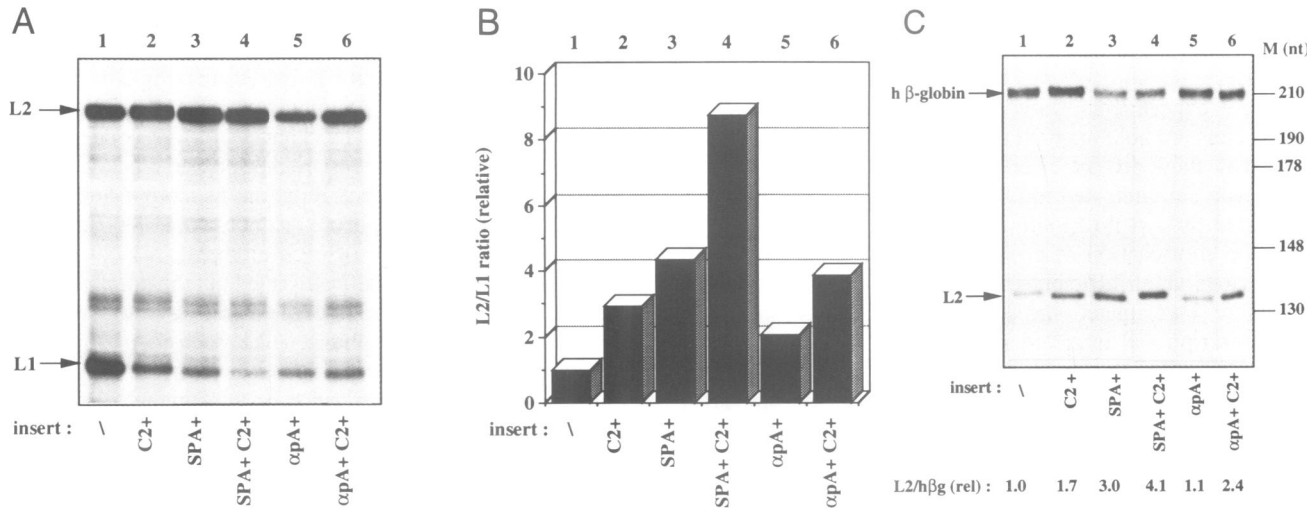

Fig. 5. Additive effect of poly(A) sites and the C2 pause site on promoter protection. (**A**) RNase protection assay. HeLa cells were transfected with the parental L1αL2α plasmid and with derivatives containing either a single element (SPA+ or αpA+ in the Asp 718 site; C2+ pause site in the *Sca*I site) or a combined element (SPA+ C2+ or apA+ C2+). Cytosolic RNA was prepared and analysed as in Figure 2A. Lane 1: L1αL2α plasmid; lane 2: plasmid with C2 in sense (C2+) orientation; lane 3: with SPA in sense orientation (SPA+); lane 4: with SPA and C2 in sense orientation; lane 5: with αpA in sense orientation (αpA+); lane 6: with αpA and C2 in sense orientation. The data shown are representative of three independent experiments. (**B**) The autoradiograph in (A) was scanned with a laser densitometer to quantify the L1 and L2 signals, and to calculate the ratio of L2 to L1. The ratios were then normalized with the L2/L1 value of the L1αL2α plasmid taken as 1. (**C**) S1 nuclease analysis. HeLa cells were transfected with the parental L1αL2α plasmid or derivatives containing SPA, αpA, C2 pause site or combinations thereof, as indicated, together with a human β-globin co-transfection control. Cytosolic RNA was prepared and analysed by means of an S1 nuclease assay using a 5′ end-labelled probe to detect L2 transcripts and a 3′ end-labelled probe for the co-transfection control. The S1 data were quantified by laser densitometry to calculate the fold increase in L2 level relative to the co-transfection control. The corresponding values [L2/hβg (rel)] are given below each lane.

the mapping of nuclear RNAs by means of reverse transcriptase−PCR, have shown that pol II transcription terminates downstream of this pause site (Enriquez-Harris *et al.*, 1991; Owczarek *et al.*, 1992). The C2 pause site is found 60 nucleotides 3′ of the poly(A) site of the C2 gene in the MHC class III locus. Again, nuclear run-off data indicate that termination takes place downstream of the C2 pause site (Ashfield *et al.*, 1991). However, the actual mechanism whereby these sites contribute to the termination process is unknown. They may be pause sites that have no effect on termination unless preceded by a functional poly(A) site. Alternatively, they may act as terminators independently of a poly(A) signal. We tested these pause sites in the interference assay, individually and in combination with a poly(A) signal (see the next section).

The α pause and the C2 pause fragments were cloned in the L1αL2α interference plasmid in the *Sca*I site between LTR1 and LTR2 (see Figure 1), and their effect on promoter occlusion was analysed by means of an RNase protection and an S1 nuclease assay. As described in the previous section, RNase protection data are used to estimate the degree of promoter protection by means of the L2/L1 ratio, whereas the S1 nuclease data measure changes in L2 levels relative to the co-transfection control. In the RNase protection assay (Figure 4A and B), both pause sites were able to increase the L2/L1 ratio, indicating that a single pause site is sufficient to prevent occlusion of the downstream LTR2 promoter. The pause sites acted in an orientation-independent way, but their effect was sequence specific as a control plasmid containing a pGem7-derived spacer fragment of approximately the same length in the *Sca*I site had no effect on the L2/L1 ratio. Similar results were obtained in the S1 nuclease assay (Figure 4C) where each pause site caused an increase in the L2 signal, again independently of their orientation. Previous data indicated that these elements acted preferentially in the sense orientation when tested in an indirect assay for pol II termination based on a competition between closely spaced poly(A) sites (Ashfield *et al.*, 1991). However, the orientation dependency in the poly(A) site competition assay was only partial as the antisense pause sites were still active, albeit less than in the sense orientation. Also, the poly(A) site competition and the interference assays measure different parameters [poly(A) site usage versus transcriptional activity of an occluded promoter] and it may well be that for reasons not understood at present the orientation of pause sites is a critical parameter for poly(A) site usage, but less so for blocking transcriptional interference.

Additional control experiments showed that the increase in the L2 signal could not be ascribed to an enhancer effect of the C2 or α pause site on the LTR promoter. When cloned upstream of LTR2 in a single transcription unit vector, neither the α nor the C2 pause site had a significant effect on the L2 signal (see Figure 4D, lanes 1−4). Neither did the C2 fragment affect mRNA stability, as shown by the L2α and L2α C2+ lanes in Figure 4D. Unexpectedly, the α pause fragment led to a decrease in the mRNA level when present downstream of the LTR and contained within the transcription unit (lanes 5 and 7 in Figure 4D). One possible explanation for this observation is that the α pause site affects mRNA stability, but we have not addressed this problem further. However, it means that the increase in L2/L1 ratio observed for the α pause site constructs is only partially due to a promoter protection effect. That the α pause site still has a genuine promoter protection effect is demonstrated by the S1 analysis in Figure 4C, where the L2 signal of the α pause constructs is increased when compared with the spacer control (Figure 4C, lanes 4−6). As the α pause site is cloned upstream of the cap site of the LTR2 promoter,

it is not contained within the LTR2 transcripts. L2 signals are therefore not affected by α pause effects on RNA stability.

These experiments show that both the α and the C2 pause sites are able to alleviate transcriptional interference, and that they do not need a poly(A) signal to do so. However, in their natural context both pause sites are preceded by a poly(A) site and it has been proposed that it is the combination of a poly(A) signal and a pause site that brings about efficient transcriptional termination. We therefore investigated the effect of such a combination on transcriptional interference.

Promoter protection by poly(A) signals is potentiated when followed by the C2 pause site

To examine the effect of poly(A) signal + pause site combinations on transcriptional interference, we modified the L1α-SPA(+)-L2α and L1α-αpA(+)-L2α plasmids by cloning the C2 pause site in the sense orientation downstream of the poly(A) sites in the *Sca*I site (see Figure 1). These constructs were then analysed by means of an RNase protection and an S1 nuclease assay as previously described.

The results of the RNase protection assay are shown in Figure 5A and B. Whereas the C2+ pause site and the SPA raised the L2/L1 ratio 3- and 4-fold, respectively, the SPA+ C2+ combination increased the L2/L1 ratio ~8-fold. Similarly, the αpA+ C2+ combination resulted in a stronger effect than either αpA+ or C2+ pause site alone. Qualitatively similar results were obtained in the S1 nuclease experiment (Figure 5C). As the promoter protection effect of combined elements exceeds that of individual elements, this suggests that poly(A) signals and pause sites work additively to block transcriptional interference. These data also imply that a weak poly(A) signal such as the α2-globin poly(A) site, which on its own is ineffective in preventing promoter occlusion, requires an additional downstream pause site for efficient alleviation of interference.

Discussion

Transcriptional interference or promoter occlusion is a potential problem for genes that are relatively closely spaced, but it can be overcome by efficient transcriptional termination in the intergenic region, as demonstrated for the pol I rRNA transcription units (Bateman and Paule, 1988; Henderson *et al.*, 1989; McStay and Reeder, 1990). We have now addressed the question of how pol II termination signals affect transcriptional interference of pol II promoters. Using an interference assay based on two retroviral LTRs in tandem, we were able to conclude that: (i) both components of a pol II termination signal, a poly(A) signal and a transcriptional pause site, can block interference of a downstream promoter; (ii) the protective effect of a combined element comprising a poly(A) signal followed by a pause site exceeds that of either site alone; (iii) the interference-blocking effect of a poly(A) signal correlates with its strength as a processing site with a stronger poly(A) site being a more potent blocker of promoter occlusion. Although not addressed in these experiments, it is conceivable that the activity of a transcriptional pause site can also be modulated. The C2 fragment which we used in this study contains a binding site for the recently cloned MAZ protein (Ashfield *et al.*, 1991; Bossone *et al.*, 1992). Furthermore, MAZ binding is necessary for the pausing activity of the C2 fragment as determined in a poly(A) site competition assay (Ashfield *et al.*, 1991). Thus, it is possible that C2 pausing activity could be regulated by changes in MAZ concentration or activity. The efficiency of an interference-blocking signal will therefore depend not only on its composition, i.e. whether it consists of a poly(A) signal, a pause site or both, but also on the strength of each element.

Although we have specifically ruled out the possibility that the C2 and α pause sites stimulate transcription from the downstream LTR in an enhancer-like mechanism, this is not necessarily true for every *cis*-active element involved in pol II transcriptional termination or in promoter protection. Indeed, some factors may act as enhancers and terminators at the same time. For example, it was found that an inverted CCAAT box upstream of the adenovirus major late promoter (MLP) directed transcriptional termination and consequently prevented occlusion of the MLP (Connelly and Manley, 1989a,b). However, not all protein-binding sites on the DNA are capable of preventing interference or causing termination. For example, in the same study Sp1 sites did not induce termination and neither did an SV40 enhancer fragment (Connelly and Manley, 1989b). Similarly, the HIV-LTR fragment used in this study contains a TATA box, two NF-κB and three Sp1 sites. HeLa cells or HeLa cell extracts have been shown to contain the cognate factors that recognize and bind to these sites in the HIV promoter (Garcia *et al.*, 1987; Clark *et al.*, 1990). Yet the HIV-1 promoter is still sensitive to occlusion when transfected into HeLa cells. Why some DNA-binding factors are capable of inducing termination and/or preventing interference, whereas others are not, is still a matter of conjecture. Conceivably, differences in stability of the DNA–protein complex or specific modes of interaction between the DNA-bound factor and the transcription complex may play a role.

What are the implications of these results for transcriptional interference between closely spaced genes? An important question in this respect is over what distance an interference effect can be transmitted. In the ALV retrovirus, the 5′ and 3′ LTR are several kilobases apart (Cullen *et al.*, 1984). Similarly, in the L1αL2α plasmid where LTR2 interferes with LTR1 (see Figure 2), the distance between the poly(A) site of the LTR2 transcript and the cap site of LTR1 is ~3.5 kb. This is of the same order of magnitude as the intergenic distance in some gene clusters: e.g. in the MHC class III locus, the distance between the poly(A) site of the C2 gene and the cap site of the next gene, factor B, is only 421 nucleotides (Wu *et al.*, 1987) and several other genes in this locus are separated by only a few kilobases; also, in the human α-globin locus the distance between the α2 poly(A) site and the cap site of the α1 globin gene is 4.4 kb. Consequently, interference is a possibility in these situations and it must be overcome if the 5′ and 3′ genes are to be expressed in an independent manner. Based on our experiments, we predict that independent expression of closely spaced genes requires an interference-blocking element in the intergenic region. This element can, as shown in this study, consist of a strong poly(A) signal, a transcriptional pause site or, ideally, both.

Another possible scenario for transcriptional interference is between tandem promoters driving the expression of the

same gene (e.g. the *Drosophila adh* gene; Corbin and Maniatis, 1989) or of nested genes (e.g. the adenoviral E1B-polypeptide IX genes; Vales and Darnell, 1989). Initiation from the downstream promoter will only be possible either when the upstream promoter is inactive (as is the case in the *adh* and adenovirus situation) or, alternatively, in the presence of a suitable interference-blocking signal which would prevent an active upstream promoter from interfering with the downstream promoter. This interference-blocking signal could consist of a 'classical' promoter or enhancer element with the additional capability of inducing transcriptional termination, such as the inverted CCAAT box (Connelly and Manley, 1989a,b), or of a pause site without enhancer activity similar to those we have analysed. Interestingly, the human and the murine c-*myc* genes have two principal promoters, P1 (upstream) and P2 (downstream), with a MAZ binding site (previously called ME1a1) lying in between them [for a review of c-*myc* gene expression, see Marcu *et al.* (1992)]. In CV1 cells transfected with a c-*myc* gene, both P1 and P2 are active with the downstream promoter P2 being predominantly used. Deletion of the MAZ site downregulates P2 activity and upregulates P1 expression (Bossone *et al.*, 1992). These observations are consistent with the MAZ site acting as an interference-blocking site that permits initiation at P2 in spite of an active P1 promoter. Results compatible with such an interference-blocking role for MAZ in the c-*myc* promoter were also obtained in a *Xenopus* expression system (Meulia *et al.*, 1992). However, in another series of experiments also performed in a *Xenopus* expression system, deletion of the MAZ site had no effect on termination of P1 transcripts (Roberts *et al.*, 1992).

Our data may also have some implications for situations in biotechnology where the expression of two or more proteins from one single plasmid in mammalian cells is required. Our results indicate that for maximal levels of expression one should not only rely on powerful promoters, but one should also minimize the risk of transcriptional interference by inserting a potent interference-blocking element, i.e. a strong poly(A) site such as SPA, followed by a transcriptional pause site, downstream of each transcription unit. A strong poly(A) site will be advantageous in two ways. Not only will it protect the downstream gene from interference and hence increase its expression, but it will also increase the expression of the upstream gene because of the established link between poly(A) site strength and cytosolic mRNA levels (Gil and Proudfoot, 1987).

In this study, we have used sequence elements which are thought to play a role in pol II termination. The interference-blocking effect of poly(A) signals and pause sites, as described in these studies, is certainly consistent with their proposed role in termination. Moreover, the observed effect of poly(A) site strength on promoter protection supports the hypothesis that strong poly(A) sites will increase the efficiency of the termination process. However, the situation is less clear for the pause sites. As already mentioned, it is still unknown whether these elements can only retard the pol II complex or whether they act as proper terminators on their own. Unfortunately, the interference assay does not allow us to discriminate between either possibility. One can argue that for promoter protection to occur, as measured in these experiments, termination *per se* is not required and that pausing, i.e. a temporary block to polymerase passage, may be sufficient. More direct experiments, such as nuclear run-off analysis or the mapping of nuclear transcripts, will be required to resolve this issue.

Materials and methods

Plasmid constructs

A pSP65 vector containing the minimal HIV1-LTR (starting at *Ava*I in U3 and ending at *Hin*fI in U5; this region contains all the elements required for efficient transcription in HeLa cells; see Garcia *et al.*, 1987) in the *Sma*I site was opened with *Bam*HI 3′ of the HIV-LTR. A fragment of the human α2-globin gene extending from the *Sma*I site in intron 1 to the *Bst*EII site in exon 3 was inserted to generate pSP-L1α. Another α2-globin fragment going from the *Nco*I site in exon 1 to an *Xba*I site (polylinker-derived) 3′ of the α2 poly(A) site was inserted in the same *Bam*HI site to create pSP-L2α. The L1α unit was then cut with *Sca*I and *Xba*I, blunt ended with Klenow enzyme and cloned in the *Xho*I site (filled in) of the pGEM7-Zf(+) polylinker to give pGEM-L1α. An *Eco*RI−*Xba*I fragment containing the L2α unit was then inserted in pGEM-L1α opened with *Cla*I and *Bam*HI to create pGEM-L1αL2α. The dual transcription unit, L1αL2α, was then transferred as a blunt-ended *Xba*I−*Mlu*I fragment to the pSVod expression vector (cut with *Eco*RI and *Ase*I, and blunt ended) to generate the pSVod-L1αL2α expression plasmid. The pSVod vector is described by Proudfoot *et al.* (1992).

Expression vectors containing single transcription units, pSVod-L1α and pSVod-L2α, were synthesized by taking the L1α unit (*Xba*I to *Nco*I) or the L2α unit (*Eco*RI to *Mlu*I) from pGEM-L1αL2α and ligating them as blunt-ended fragments in the *Eco*RI−*Ase*I-cut pSVod vector.

Derivatives of pSVod-L1αL2α were obtained by blunt-end ligation in the unique *Asp*718, *Sca*I or *Mlu*I sites. The SPA and the α2-poly(A) site fragments were cloned in the *Asp*718 site. The C2 and α pause fragments, and the spacer control fragment, were inserted in the *Sca*I site. A combined element consisting of SPA followed by the C2 pause site was also cloned in the *Mlu*I site. Promoter deletion constructs were created by cutting out LTR1 or LTR2 from the pSVod-L1αL2α plasmid followed by religation. Deletion of LTR1 (pSVod-ΔL1αL2α) was obtained by an *Xba*I−*Afl*II (partial) digest, deletion of LTR2 (pSVod-L1αΔL2α) by an *Sca*I−*Pvu*II (partial) digest. Derivatives of pSVod-L2α were obtained by cloning SPA, C2 or α pause fragments in the *Tth*III1, the *Eco*RI or the *Sca*I site of pSVod-L2α. The SPA is a previously characterized 54mer oligonucleotide encoding a strong poly(A) site (Levitt *et al.*, 1989). The α2-poly(A) site was an *Eco*0119−*Xba*I fragment taken from the α2W3′PSΔ3SPA vector (Ashfield *et al.*, 1991). The C2 pause site was a *Sty*I−*Bam*HI fragment from the pMLC2.B vector (Ashfield *et al.*, 1991). The α pause site was an *Hpa*I−*Bgl*II fragment from the SΔ3 vector (Enriquez-Harris *et al.*, 1991). The spacer fragment was a 105 nucleotide *Hae*III fragment from pGEM7-Zf(+).

All constructs were checked by restriction enzyme mapping and/or sequencing. DNA manipulations were performed as described by Sambrook *et al.* (1989).

Transfection procedures

HeLa cells (150 mm diameter dishes; 50% confluent) were transfected with 4 μg of the various plasmids using the calcium phosphate precipitation technique. A Tat expression plasmid (Adams *et al.*, 1988) to transactivate the HIV-1 LTR, and a human β-globin expression plasmid (Proudfoot *et al.*, 1992) to control for transfection efficiency, were always co-transfected. The medium was changed after 10 h. Cells were harvested 48 h after transfection and cytosolic RNA prepared as described by Sambrook *et al.* (1989). Briefly, cells were lysed in a 500 μl NP-40 lysis buffer and nuclei were removed by spinning them through a 500 μl sucrose cushion. The upper layer (500 μl) was twice phenol−chloroform extracted, ethanol precipitated and re-suspended in H_2O. One-fourth to one-fifth of the cytosolic RNA was used for RNase protections or for S1 nuclease assays. The integrity of the RNA was checked by running an aliquot on a 1% agarose gel.

RNase protection analysis

A restriction fragment (*Ava*I−*Ava*I from pSP-L2α) spanning the LTR2 and the first part of the α2-globin gene up to the *Sma*I site in intron 1 was subcloned in a pGEM7-Zf(+) vector. The DNA was cut with *Pvu*II and 0.5 μg of the linearized template was *in vitro* transcribed in the presence of [α-^{32}P]CTP (800 Ci/mmol) with T7 RNA polymerase (Promega) for 1 h at 37°C according to the manufacturer's instructions. The DNA

template was then digested with DNase I, RNase-free (Boehringer), and the RNA probe was purified by a phenol/chloroform extraction and then ethanol precipitated.

RNase protections were carried out essentially as described in Sambrook *et al.* (1989). Briefly, one-fourth or one-fifth of the cytosolic RNA was hybridized with 1/1000 of the antisense RNA probe (corresponding to 500 c.p.s. on a hand-held Geiger counter) in 30 μl of formamide 80%, PIPES 40 mM (pH 6.8), NaCl 0.4 M and EDTA 1 mM. After an overnight incubation at 56°C, 300 μl of an RNase digest mix [Tris−HCl 10 mM (pH 7.4), NaCl 0.3 M, EDTA 5 mM, RNase A 40 μg/ml and RNase T1 1000 U/ml) were added and RNA digestion was performed at 30°C for 45 min. Finally, the samples were treated with proteinase K, phenol/chloroform extracted and ethanol precipitated. The protected RNA fragments were then analysed on a denaturing 4% polyacrylamide/8M urea gel. Autoradiographs were exposed at −70°C for 24−48 h. The autoradiographs were then quantified by laser densitometry scanning.

S1 nuclease mapping

S1 probes were generated by 5′ or 3′ end-labelling of appropriate restriction fragments essentially according to Sambrook *et al.* (1989). The L2 5′ end probe was made by cutting the pSP-L2α plasmid with *Nco*I and dephosphorylating it with alkaline phosphatase. The digest (500 ng) was 5′ labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol). The human β-globin 3′ probe was obtained by cutting the β-globin plasmid with *Eco*RI. Then 500 ng of the digest were used to fill in the 3′ ends with Klenow enzyme and [α-^{32}P]dATP (3000 Ci/mmol). Probes were separated from the non-incorporated label via a Sephadex G-100 column.

One-fourth or one-fifth of the cytosolic RNA was hybridized with ~1 ng of the 5′ or 3′ labelled probes in 30 μl of the same buffer as used for RNA protections. After an overnight incubation at 52°C, 300 μl of an S1 digest mix [NaCl 0.28 M, Na-acetate 50 mM (pH 4.5), $ZnSO_4$ 4.5 mM, denatured salmon sperm DNA 20 μg/ml and S1 nuclease 800 U/ml] were added and S1 nuclease digestion was performed at 30°C for 1 h. The S1 digest was stopped by adding 100 μl of S1 stop buffer (EDTA 50 mM, NH_4-acetate 4 M and tRNA 200 μg/ml) and ethanol precipitated. The protected DNA fragments were then analysed on a denaturing 4% polyacrylamide/8 M urea gel. Autoradiographs were exposed at −70°C for 24−72 h. The autoradiographs were quantified by laser densitometry scanning.

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