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Multiple Transcript Cleavage Precedes Polymerase Release in Termination by RNA Polymerase II

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Summary

The requirement of poly(A) signals to elicit transcription termination of RNA polymerase II (pol II) is firmly established. However, little else is known about the actual process of pol II transcription termination. Evidence presented in this paper, based on analysis of nascent transcripts of the human β- and ε-globin genes, demonstrates that pol II transcription termination involves two distinct and temporally separate events. The first event, termed pretermination cleavage (PTC), is mediated by sequence tracts located downstream of the poly(A) site which appear to promote heterogeneous cleavage of the nascent transcript. The second event, in which pol II disengages from the DNA template, requires that polymerase has transcribed both a PTC sequence tract and a functional poly(A) site.

Introduction

Transcription termination is an important process as it enhances gene expression by facilitating polymerase recycling and thus maintains a pool of available polymerase (Dieci and Sentenac, 1996; Dye and Proudfoot, 1999). Moreover, termination prevents interference at downstream promoters (Greger and Proudfoot, 1998) and may also enhance the energy balance of the cell by reducing wasteful transcription of long tracts of noncoding sequences.

For transcription termination to take place, both release of the RNA transcript from polymerase and dissociation of polymerase from the DNA template must occur. These events have been described for genes transcribed by *E. coli* RNA polymerase (Yarnell and Roberts, 1999), RNA polymerase I and III (Paule and White, 2000). Although there are significant differences between these systems, each has been analyzed at the molecular level and is therefore understood in some detail (reviewed by Platt, 1998; Uptain et al., 1997).

In contrast, the mechanism of transcription termination by RNA polymerase II (pol II) is poorly understood. The reason for this is that although pol II transcription termination defines the 3′ end of the primary RNA transcript, the transcript itself is cleaved at the poly(A) site. In a recent study, it was shown that cleavage at the poly(A) site occurs at the same time as or just before transcription termination (Dye and Proudfoot, 1999). Cleavage of the nascent transcript at the poly(A) site leaves two products which have quite different fates; the 5′ (upstream) product, destined to become mRNA, is stabilized by the addition of the poly(A) tail, whereas the 3′ (downstream) product is destabilized by cleavage which separates it from the body of the transcript. The 3′ cleavage product extends up to the site of transcription termination, which may lie thousands of base pairs (bp) downstream of the cleavage site (Proudfoot, 1989). It is not possible to confidently map the extent of this now uncapped nascent transcript by standard transcript mapping techniques because it is very rapidly degraded.

The surest way of measuring transcription termination is to label the nascent transcript during synthesis using the nuclear run on assay (NRO), and so determine sites of active transcription. Using this technique, it was found that pol II transcription termination is a somewhat random process occurring at sites between 200–2000 bp downstream of the poly(A) site, but is dependent upon the presence of a functional poly(A) signal (Proudfoot, 1989). Since then, attention has focused on the coupling of RNA processing and transcription termination. In the yeast *S. cerevisiae*, it has been shown that termination is entirely dependent upon the cleavage/polyadenylation factors. It has also been demonstrated that in mammalian nuclei, the dependence of termination on RNA processing extends to splicing of the terminal intron. These and other data provide strong evidence for the notion of cotranscriptional RNA processing carried out by a pol II transcription "factory" (Proudfoot, 2000; Cook, 1999).

Although RNA processing signals are major players in pol II transcription termination, several reports indicate that sequences downstream of the poly(A) site are also required (Tantravahi et al., 1993; Birse et al., 1997; Proudfoot, 1989). In the present study, the role of sequences located downstream of the human β- and ε-globin poly(A) sites (3′ flanking regions) in transcription termination is examined. Nascent transcript analysis shows that 3′ flanking region sequences that are required for efficient transcription termination mediate a transcript cleavage activity, herein termed pretermination cleavage (PTC), that effectively releases the nascent transcript from the elongating polymerase. It is proposed that PTC, which precedes polymerase release from the DNA template, is required for transcription termination. Interestingly, PTC is not dependent on the presence of a functional poly(A) signal, whereas the subsequent polymerase release step is. These data indicate that pol II transcription termination occurs through at least two spatially and temporally distinct processes.

Results

NRO Analysis of the β-Globin Gene in Chromosomal and Plasmid Contexts

Transcription termination in general occurs between a few hundred bp to several kilobase pairs (kbp) downstream of the poly(A) site (Proudfoot, 1989). Here, the extent of nascent transcription on the human β-globin gene was determined by NRO analysis of spleen cells from transgenic mice containing the entire human β-globin

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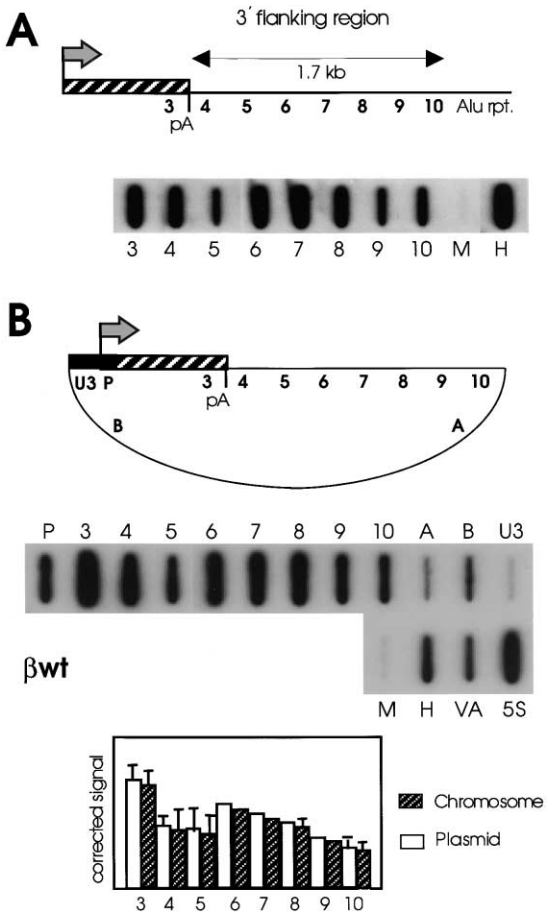

Figure 1. NRO Analysis of the Human β-Globin Gene and 3′ Flanking Region

(A) NRO analysis of human β-globin gene transcription in a transgenic mouse containing the human β-globin LCR. In the diagram of the human β-globin gene and 3′ flanking region (not drawn to scale), the arrow indicates the start site of transcription from the β-globin promoter. The β-globin gene is represented by the hatched box. pA indicates the position of the poly(A) site. Characters in bold type indicate the position of NRO probes. Probe M is M13 DNA and controls for background hybridization. Probe H contains histone DNA and is a positive control for pol II transcription.

(B) NRO analysis of HeLa cells transiently transfected with the βwt plasmid. In the diagram (not drawn to scale) of the βwt plasmid, the HIV LTR is represented by a filled box. The 5S probe contains 5S rDNA and measures pol III transcription. The VA probe detects transcription from a cotransfected control plasmid containing the adenovirus *VAI* gene. Signals obtained from NRO analysis in (A) and (B) were normalized for background hybridization and incorporation of α^{32}P-UTP before plotting on the graph below the NRO data.

LCR (Figure 1A; Strouboulis et al., 1992). The hybridization signals detected over all of the numbered single-stranded DNA probes, representing the first 1.7 kb of the β-globin 3′ flanking region, indicate that pol II is transcriptionally active up to at least 1.7 kb downstream of the β-globin poly(A) site. Probe M is a control for background hybridization and probe H is an internal positive control that detects transcription of the histone H4 gene. More distal areas of the β-globin 3′ flanking region have not been probed as analysis is complicated by the presence of Alu repeats and intergenic transcripts (Ashe et al., 1997). To ensure that the 3′ flanking region hybridization signals are not due to cross hybridization with cellular RNA, NRO analysis was also carried out on a wild-type mouse. In this analysis, no transcripts hybridized to the 3′ flanking region probes (K. Plant and N.J.P., unpublished data).

In order to analyze the role of the β-globin 3′ flanking region in pol II transcription termination, it was necessary to move to a transient transfection system that is more amenable to mutational analysis. Figure 1B shows NRO analysis of HeLa cells transiently transfected with a construct, labeled βwt, which bears the human β-globin gene driven by the Tat-inducible HIV LTR. In the presence of the *trans*-activating factor Tat (Cullen, 1993), nascent transcripts are detected at high levels throughout the β-globin 3′ flanking region. Quantitation of NRO signals (by phosphoImager analysis and normalization for incorporation of radiolabeled UTP) from the human β-globin gene in either the chromosomal context of the transgenic mouse or on the plasmid, in transfected HeLa cells, shows that the polymerase distribution in the 3′ flanking region is almost identical (see graph Figure 1B). Furthermore, NRO analysis of βwt plasmid indicates that the 1.7 kb of the β-globin 3′ flanking region present is sufficient to induce pol II transcription termination. This is revealed by the fact that pol II does not transcribe into the plasmid probes A, B, and U3, as demonstrated by the low hybridization signals on these probes. It therefore follows that results obtained using the plasmid borne β-globin gene are relevant to the human β-globin gene in a natural chromosomal context.

Sequences in the 3′ Flanking Region Mediate Transcription Termination

To test the dependence of pol II transcription termination on sequences in the human β-globin gene 3′ flanking region, construct βΔ5–10 was made in which all but the first 200 bp of the 3′ flanking region were removed from plasmid βwt (see diagram in Figure 2A). In NRO analysis of βΔ5–10, shown in Figure 2A, hybridization signals are detected over the plasmid probes A, B, and U3, indicating that the 3′ flanking region plays an important role in transcription termination. Corrected hybridization signals from this experiment are displayed in the graphs in Figures 2D and 2E.

To localize the transcription termination sequences, two subclones of βwt were tested that delete either regions 5–7 or regions 8–10 of the 3′ flanking region (Figure 2B). NRO analysis of βΔ5–7 shows only background hybridization signals over probes A, B, and U3, indicating that the remaining 3′ flanking region sequences (region 8–10) support full levels of transcription termination. The 5′ end of the 3′ flanking region is not required for efficient transcription termination. In contrast, NRO analysis of βΔ8–10 indicates that read-through transcripts are detected over the plasmid probes A, B, and U3, confirming that regions 8–10 are required for transcription termination. These sequences are therefore referred to as the 8–10 termination region. (Note that the weak signal over probe 5 is an artifact caused by the formation of an atypically weak RNA:DNA hybrid [Dye, 1999]).

Hybridization signals detected over probes A, B, and U3 have previously been shown to be indicative of non-terminated pol II transcribing around the entire plasmid following mutation of mRNA processing signals (Dye and Proudfoot, 1999). To control for the possibility that

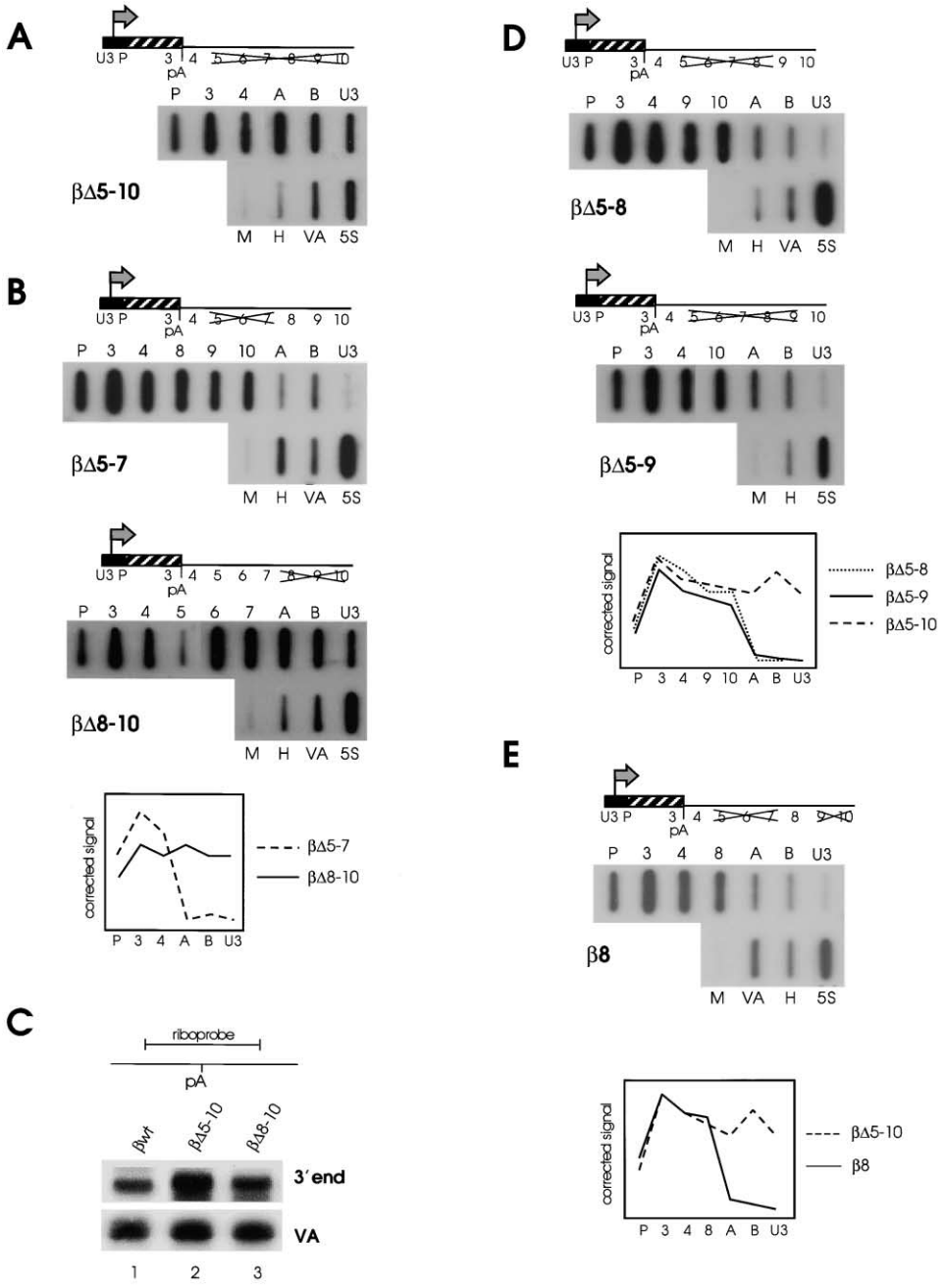

Figure 2. Transcription Termination Sequences in the β-Globin 3′ Flanking Region

(A) NRO analysis of βΔ5–10 plasmid. In the diagram of the βΔ5–10 plasmid, the crossed lines indicate the deleted part of the 3′ flanking region. Corrected hybridization signals are shown in the graphs in (D) and (E).

(B) NRO analysis of βΔ5–7 and βΔ8–10 plasmids. Corrected hybridization signals are shown in the graph below the NRO data.

(C) RNase protection analysis of βwt., βΔ5–10, and βΔ8–10 transcripts. A diagram of the RNase protection probe is shown. The VA signal derives from the cotransfected VAI plasmid and controls for transfection efficiency and RNA recovery.

(D) NRO analysis of βΔ5–8 and βΔ5–9 plasmids. Corrected hybridization signals are shown in the graph below the NRO data.

(E) NRO analysis of β8 plasmid. Corrected hybridization signals are shown in the graph below the NRO data.

the 3′ flanking region deletions affect mRNA processing and therefore only indirectly affect transcription termination, RNase protection analysis was carried out on βΔ5–10 and βΔ8–10 transcripts (see Figure 2C). The accurate processing and unimpaired accumulation of βΔ5–10 and βΔ8–10 transcripts indicate that mRNA processing is unaffected by the 3′ flanking region deletions. This experiment confirms that the β-globin 3′ flanking region directly affects pol II transcription termination.

Next, the termination activity of the 8–10 region was mapped more precisely. NRO analysis was performed on two deletion constructs, βΔ5–8 and βΔ5–9, from which either the first 300 bp (corresponding to probe 8) or 460 bp (corresponding to probe 8/9) of the termination

region were removed (Figure 2D). The low hybridization signals over the plasmid probes A, B, and U3 indicate that transcription termination is unaffected by either of these deletions. Finally, the 5′ end (region 8) of the 8–10 termination region was tested for termination activity by NRO analysis of a construct labeled β8 (Figure 2E) and shows low hybridization signals over probes A, B, and U3. Comparison of the corrected βΔ5–10 and β8 NRO hybridization signals indicate that region 8 can independently mediate transcription termination. Considering that in vivo region 8 is the first to be encountered by pol II, this is likely to be the critical termination element in the physiological system.

Transcription Termination Sequences Mediate Transcript Cleavage

A refinement of the NRO protocol, termed hybrid selection NRO analysis, enables magnetic separation of nascent transcripts on the basis of their complementarity to a biotinylated RNA probe. Using this technique, it was previously shown that 3′ flanking region transcripts, which are continuous with the protein coding region of the β- and ε-globin genes, can extend over 1 kb downstream of their respective poly(A) sites (Dye and Proudfoot, 1999). It was further noted that the extent of selected uncleaved β-globin transcripts is coincident with the 5′ end (region 8) of the 8–10 termination region. This observation prompted a further investigation into the determinants of the maximal length of selected transcripts within the β-globin 3′ flanking region. Hybrid selection NRO analysis was performed on βΔ5–7 transcripts using a biotinylated selection probe (Bio β4) complementary to the sequence immediately downstream of the β-globin poly(A) site corresponding to NRO probe 4 (Figure 3A). Following hybridization of Bio β4 to the NRO-labeled RNA and magnetic separation, the selected and supernatant fractions were hybridized to separate NRO filters. In the selected fraction, shown in Figure 3A, hybridization signals are detected over probes 3, 4, and 8, but not over probes P, 9, and 10. Note that probe 3, which is upstream but adjacent to Bio β4, shows a hybridization signal due to polymerase transcribing into the selected RNA region during the NRO transcription reaction. Transcripts from regions located more than 100 bp upstream of the selection probe are not selected as 100 nt is the approximate limit of transcription elongation in the NRO transcription reaction (Dye and Proudfoot, 1999). Thus, the background level signal over probe P is due to its location 1.5 kb upstream of the selection probe. The hybridization signals over probes 4 and 8 show that transcripts continuous with region 4 extend into region 8. However, the background level signals over probes 9 and 10 show that these transcripts do not extend beyond region 8. The corrected hybridization signals from this analysis, plotted alongside those from the βΔ5–7 NRO experiment (see Figure 2B), are shown in the graph in Figure 3A. These results lead to an unexpected conclusion. It appears that nascent transcripts detected by probes 9 and 10 are not part of a continuous transcript that extends from the protein coding region of the gene through to the end of the termination region. To test whether this result was specific to transcription initiation from the HIV LTR, the same NRO and hybrid selection NRO analyses were carried out on a construct containing the wild-type β-globin promoter transcriptionally activated by the SV40 enhancer (Treisman et al., 1983). Identical results were obtained confirming that transcript discontinuity within the β-globin termination region is not dependent on transcription from the HIV LTR (data not shown).

This surprising result demands rigorous controls. The first of which tested the possibility that 3′ to 5′ degradation of the 9–10 region transcript occurs during the selection process. This is addressed by examination of NRO-labeled βΔ5–7 transcripts in the supernatant fraction, shown in the lower panel of Figure 3A, where hybridization signals are detected over all probes. The weaker signal over probe 4 is due to the hybridization of the majority of nascent transcripts from this region to the Bio β4 probe, and thus their removal to the selected fraction. Importantly, the presence of hybridization signals over probes 9 and 10 confirms that these transcripts are intact and have not been degraded during the hybrid selection procedure.

The second control tested for a promoter function which could give rise to a second separate transcript over probes 9–10. To this end, NRO analysis was carried out on βΔ5–7 in the absence of the *trans*-activating factor Tat (see Figure 3B). The hybridization signal over probe P, which results from abortive initiation events at the HIV LTR (Cullen, 1993), confirms the presence of the βΔ5–7 plasmid. No other transcripts from the βΔ5–7 plasmid are detected. The total absence of hybridization signals over probes 8, 9, and 10 indicates that these sequences do not function as promoters.

The possibility that the 8–10 termination region transcript is unusually unstable, or has some autocatalytic RNA processing capacity, was controlled for by hybrid selection of a synthetic RNA transcript corresponding to the βΔ5–7 3′ flanking region (Figure 3C). The synthetic transcript was hybridized to probes 4, 8, 9, and 10, either with or without prior hybrid selection, and in each case, equivalent hybridization signals over probes 9 and 10 were detected. It is apparent that transcript discontinuity in the 8–10 termination region does not result from some autocatalytic RNA processing capacity.

The final control was to use reverse transcription PCR (RT-PCR) to confirm the hybrid selection NRO results. Nascent transcripts from HeLa cells transiently transfected with βΔ5–7 were reverse transcribed using nested primers positioned within the 8–10 termination region (Figure 3D). The resulting cDNAs were then analyzed by PCR using the indicated 3′ oligonucleotide and a common 5′ oligonucleotide (labeled a) complementary to the sequence in exon 3 of the β-globin gene (upper data panel of Figure 3D). In lane ab +RT, there is a strong band representing region 8 of the termination region. However, in all other lanes, there are no bands representing longer transcripts in the 8–10 termination region. These results confirm the NRO hybrid selection data by showing that continuous nascent transcripts do not extend beyond region 8 of the β-globin 3′ flanking region. As a control, an SP6 transcript of the 8–10 termination region was reverse transcribed and amplified in a parallel experiment (lower data panel). Here, the presence of bands in all +RT lanes indicates that each primer pair can efficiently amplify cDNA copies of the 3′ flanking region transcript.

The control experiments described above confirm that the 3′ flanking region transcripts are of two types: those that extend from the protein coding region of the gene into region 8 of the termination region and those that are found within the termination region but are not con-

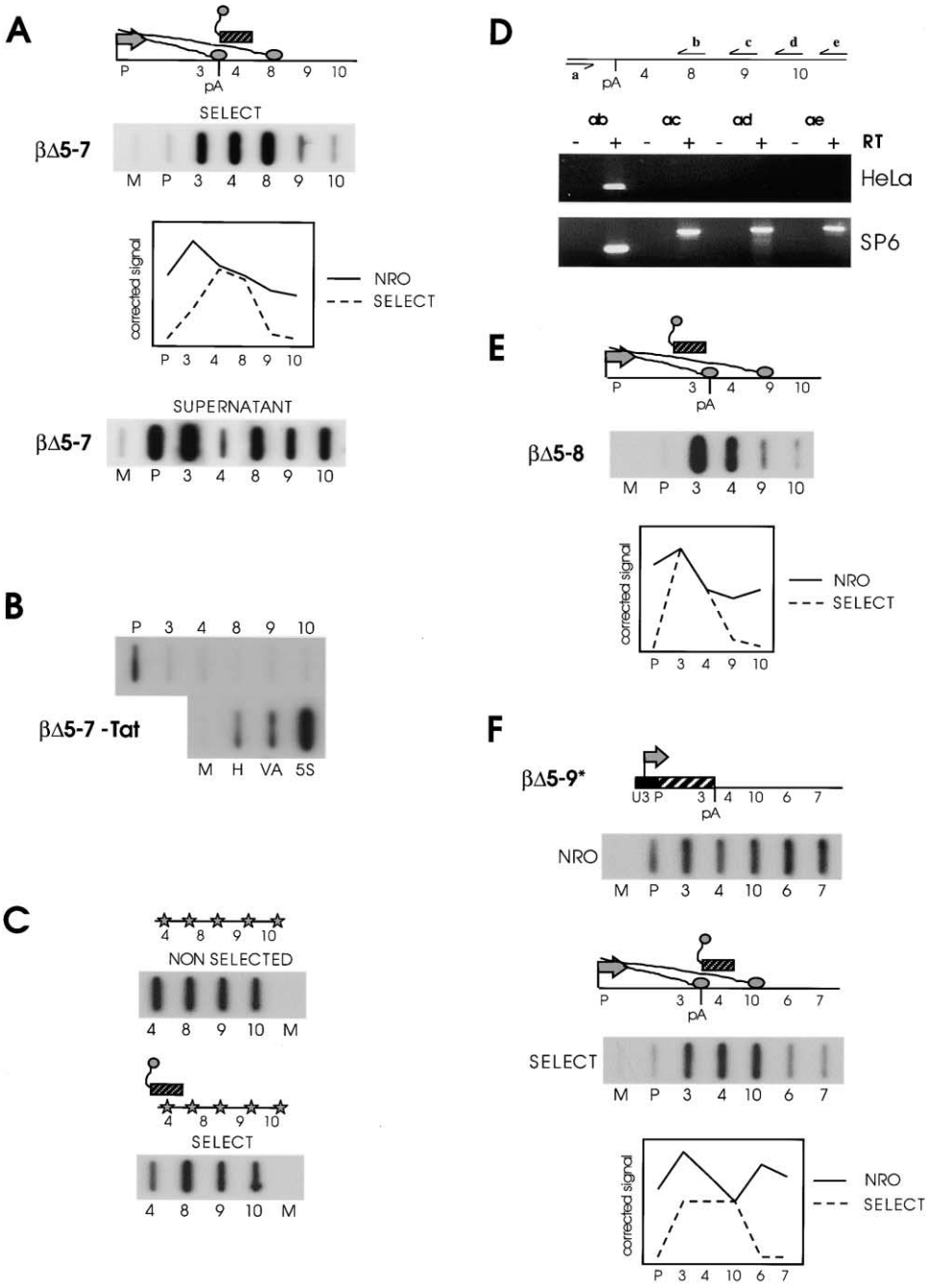

Figure 3. Transcript Cleavage in the β-Globin Termination Region

(A) Hybrid Selection NRO Analysis of βΔ5–7 transcripts. In the diagram, the position of the biotinylated probe (hatched box with tail) with respect to the βΔ5–7 3′ flanking region is shown. The filled ovals and wavy lines represent pol II and associated nascent transcripts. Corrected hybridization signals from NRO and hybrid selection NRO analyses of βΔ5–7 transcripts are shown in the graph below the NRO data.

(B) NRO analysis of βΔ5–7 plasmid in the absence of the transcriptional activator Tat.

(C) Hybrid selection analysis of a synthetic termination region transcript. In the diagrams, the radiolabeled synthetic transcript is represented by starred line.

(D) RT-PCR analysis of βΔ5–7 3′ flanking region transcripts. In the diagram of the βΔ5–7 3′ flanking region, the labeled arrows indicate the positions of oligonucleotides.

(E) NRO hybrid selection analysis of βΔ5–8 transcripts. Corrected hybridization signals are shown in the graph below the NRO data.

(F) NRO and hybrid selection NRO analysis of βΔ5–9*. Corrected hybridization signals are shown in the graph below the NRO data.

nected to upstream transcripts. It is apparent that this second type of transcript does not originate in the termination region as this sequence has no detectable promoter activity (see Figure 3B). The conclusion drawn from these experiments is that the different RNA populations in the β-globin 3′ flanking region result from tran-

script cleavage in region 8 followed by continued pol II elongation through regions 9 and 10. It appears that the cleavage event occurs cotranscriptionally and does not immediately impede the progression of pol II into regions 9 and 10 where it continues to synthesize RNA. In order to differentiate this novel transcript cleavage activity from cleavage at the poly(A) site, it is referred to as pretermination cleavage (PTC).

Testing if PTC Is Involved in Pol II Transcription Termination

It has been shown that region 8 can independently mediate pol II transcription termination (see Figure 2E) and also promotes a cotranscriptional RNA cleavage event termed PTC. In contrast, regions 5–7 of the β-globin 3′ flanking region do not mediate transcription termination (see NRO analysis of plasmid βΔ8–10 in Figure 2B) or PTC. This latter observation was made in our previous publication where the 3′ flanking region transcripts of the βwt construct (then labeled HIVβ) were shown to extend from the poly(A) site through probes 5, 6, and 7 and into probe 8 (see Dye and Proudfoot, 1999).

These results indicate that there is a functional link between PTC and pol II transcription termination. This proposition was further tested by conducting hybrid selection NRO analysis on the termination-competent βΔ5–8 and βΔ5–9 plasmids, previously examined by NRO analysis (see Figure 2D). Hybrid selection NRO analysis of βΔ5–8 transcripts, using the Bio β3 selection probe, is shown in Figure 3E. While strong hybridization signals are detected over probes 3 and 4, the hybridization signal over probe 9 is very low and that over probe 10 is only just above background level. This result indicates that the majority of transcripts are cleaved within region 9 and few extend into region 10. The corrected hybridization signals were plotted alongside those from the βΔ5–8 NRO experiment (see Figure 2D) and displayed in the graph shown below the NRO data.

To accurately map βΔ5–9 transcripts, a new plasmid labeled βΔ5–9* was made. This plasmid is a variant of βΔ5–9 modified by the addition of extra 3′ flanking sequence, regions 6 and 7 (Figure 3F), which were used as they do not appear to mediate PTC. NRO analysis of βΔ5–9*, shown in the upper data panel of Figure 3F, results in hybridization signals over all genic and 3′ flanking region probes. However, hybrid selection NRO analysis of βΔ5–9*, shown in the lower data panel of Figure 3F, results in strong hybridization signals over probes 3, 4, and 10, but only background level signals are detected over probes 6 and 7. The corrected hybridization signals from the βΔ5–9* NRO and hybrid selection NRO analysis are displayed on the graph shown below the NRO data. Comparison of the selected and nonselected NRO experiments indicates that region 10 also mediates PTC activity.

Taken together, the above observations indicate that each of the sequences of the β-globin 8–10 termination region that supports transcription termination also mediates PTC activity. These data provide strong evidence that PTC and transcriptional termination are linked events.

The Role of the Poly(A) Site in Transcription Termination

Considering the key role of RNA processing in pol II transcription termination (Proudfoot, 2000), it seems plausible that PTC could be regulated by 3′ end processing. In order to study this possibility, a variant of the βΔ5–7 plasmid, bearing a poly(A) site mutation and labeled βΔ5–7p(A)mut, was made (see diagram in Figure 4B). To confirm that the poly(A) site was inactivated in βΔ5–7p(A)mut, RNase protection mapping was carried out on nuclear RNA from βΔ5–7 and βΔ5–7p(A)mut transfections (Figure 4A). Cleavage at the poly(A) site gives a 230 nt band shown in the βΔ5–7 sample (lane 1). With βΔ5–7p(A)mut (lane 2), as expected, no band for use of the mutated poly(A) site is detected.

Next NRO and hybrid selection NRO analysis was conducted on βΔ5–7p(A)mut transcripts. In the NRO analysis, shown in Figure 4B, hybridization signals are detected in the genic and 3′ flanking regions as well as over plasmid probes A, B, and U3, confirming that transcription termination is abolished by inactivation of the β-globin poly(A) site. Interestingly, the hybrid selection NRO experiment, shown in Figure 4C, indicates that even in the absence of 3′ end processing, selected transcripts do not extend beyond region 8 of the βΔ5–7 3′ flanking region. The corrected hybridization signals were plotted alongside those from the βΔ5–7p(A)mut NRO experiment and displayed in the graph in the lower panel of Figure 4C. These data clearly show that the transcript cleavage activity is independent of RNA processing at the poly(A) site and, most importantly, that the capacity of polymerase to remain attached to the template is controlled by the poly(A) signal.

Mapping Sites of Transcript Cleavage

To map sites of PTC within the β-globin termination region at higher resolution, the NRO hybrid selection protocol was adapted by the addition of an RNase protection step (see Experimental Procedures). In brief, βΔ5–7 and βΔ5–7p(A) mut NRO transcripts and a control synthetic βΔ5–7 3′ flanking region transcript were hybridized to both a selection probe (Bio β4) and a nonradiolabeled RNase protection probe spanning regions 8–9 (see diagram in Figure 4D). After magnetic selection and RNase digestion, the protected transcripts were analyzed by PAGE. The autoradiograph in Figure 4D shows the result of this experiment. In lane 1, a protection band resulting from hybridization of the riboprobe to the full-length synthetic transcript is seen. Lane 2 (βΔ5–7 −Tat) shows the result of a control experiment in which βΔ5–7 was transfected without Tat. No bands are visible in this lane, showing that the RNase protection probe does not hybridize to cellular transcripts. In lane 3 (βΔ5–7 +Tat), protected nascent transcripts from the 8–9 termination region are seen. Because the 5′ end of the protected products is fixed, due to hybridization to the selection probe, the length of the protected products on the autoradiograph indicates the distance that the selected transcripts extend into the termination region. The length of these protected transcripts may correlate with the positions of polymerase in the NRO transcription reaction. If there were an even distribution of polymerase within region 8–9, a smear of protected products would be expected. However, a specific pattern of bands was obtained indicating that there are preferred regions of polymerase density.

A notable feature of this experiment is that the abundance of protected products decreases markedly with increasing length. This point is especially clear when one considers that longer protection products are com-

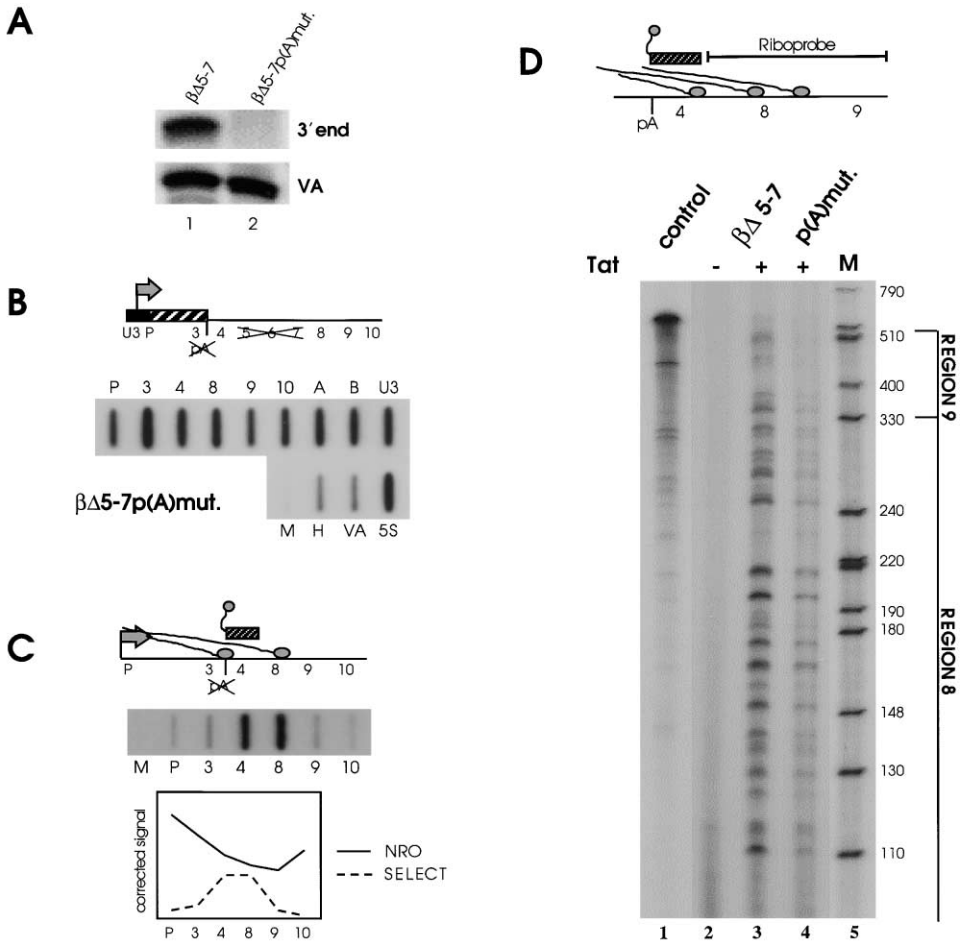

Figure 4. PTC Occurs in the Absence of mRNA 3′ End Processing

(A) RNase protection analysis of βΔ5–7 and βΔ5–7p(A)mut transcripts. The VA signal derives from the cotransfected VAI plasmid and controls for transfection efficiency and RNA recovery.

(B) NRO analysis of βΔ5–7p(A)mut plasmid. Corrected hybridization signals are shown in the graph in (C).

(C) Hybrid selection NRO analysis of βΔ5–7p(A)mut transcripts. A diagram depicting the position of the Bio β4 probe is shown above the NRO data. Corrected hybridization signals from NRO (B) and hybrid selection NRO analysis of βΔ5–7p(A)mut are shown in the graph below the NRO data.

(D) RNase protection analysis of hybrid-selected NRO-labeled βΔ5–7 and βΔ5–7p(A)mut transcripts. The diagram shows the position of the Bio β4 probe and the cold riboprobe with respect to the β-globin termination region. Molecular weight markers (M) are shown in lane 5. Sections of the autoradiograph corresponding to termination regions 8 and 9 are indicated on the bar to the right of the figure.

pressed on the gel and would therefore give relatively stronger bands than the smaller protection products. Indeed, there are few protected products beyond 330 nt, which corresponds to the 3′ end of region 8 (see guide bar to the right of the autoradiograph), and there are no full-length protection products. Therefore, this experiment confirms the NRO hybrid selection results showing that few continuous transcripts extend beyond region 8 of the β-globin 3′ flanking region. In lane 4 (βΔ5–7p(A)mut), a ladder of bands identical to those in the βΔ5–7 lane are detected. The βΔ5–7p(A)mut bands are fainter than in the βΔ5–7 lane, possibly due to lower polymerase density on the βΔ5–7p(A)mut plasmid. Importantly, there is an identical distribution of bands irrespective of the presence or absence of the poly(A) site.

Where this experiment differs from the NROs is that sites of polymerase density within the 3′ flanking region can be visualized. However, it is not possible to determine whether the positions of the bands mark the actual site(s) of PTC or are due to transient pausing of polymerase progression. If the protected products do represent sites of transcript cleavage, then it appears that PTC occurs at several sites between 110 and 330 bp into the termination region. Comparison with the control lane shows that some of the bands are aligned with degradation products of the synthetic transcript in lane 1, suggesting that these particular bands may represent degradation products of longer transcripts.

How Many Transcript Cleavage Events?

Sequences within the β-globin termination region mediate PTC, and detailed mapping of nascent transcripts indicates that multiple cleavage events occur within region 8. These observations suggest that PTC activity acts on some aspect of the overall sequence in the termination region. To determine whether PTC is coordinated solely on the basis of sequence recognition or by

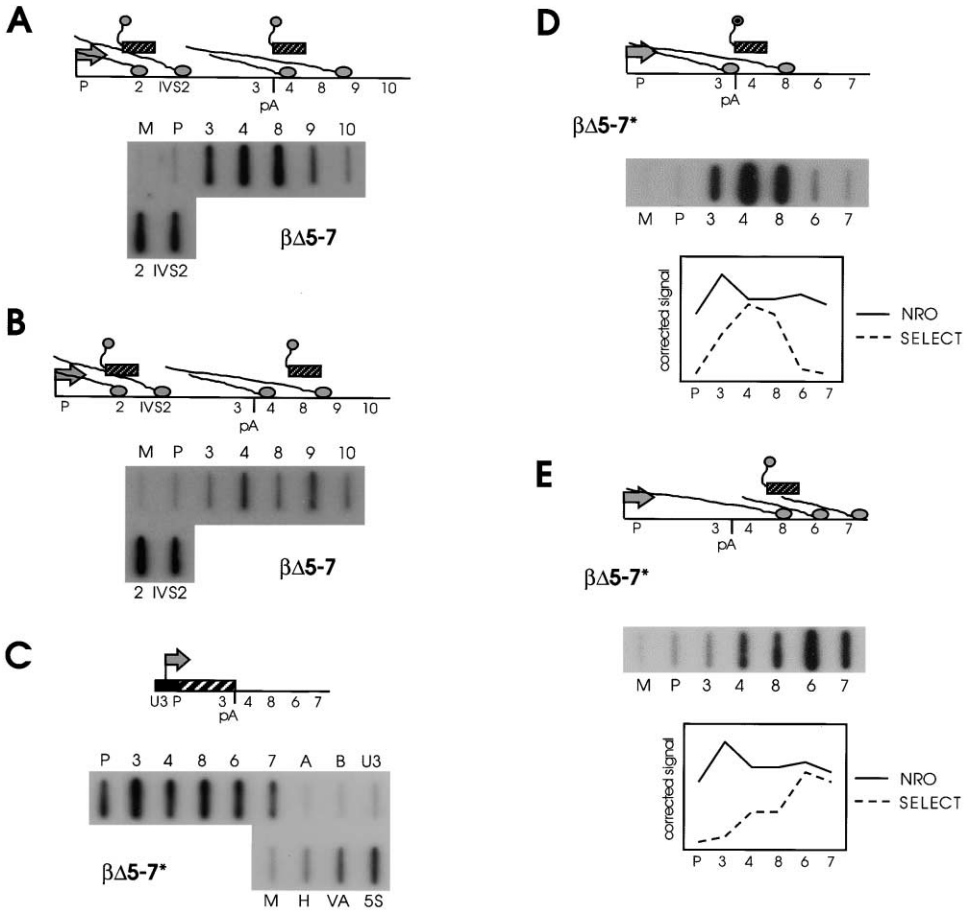

Figure 5. Analysis of Post PTC Transcripts

(A) Hybrid selection NRO analysis of βΔ5–7. The diagram shows the position of the Bio β2 and Bio β4 selection probes with respect to the βΔ5–7 3′ flanking region.

(B) Hybrid selection NRO analysis of βΔ5–7 using the Bio β2 and Bio β8 selection probes.

(C) NRO analysis of βΔ5–7*. Corrected hybridization signals are shown in the graphs in (D) and (E).

(D) Hybrid selection NRO analysis of βΔ5–7* using the Bio β4 selection probe. Corrected hybridization signals are shown with those from (C) in the graph below the NRO data.

(E) Hybrid selection NRO analysis of βΔ5–7* using the Bio β8 selection probe. Corrected hybridization signals are shown with those from (C) in the graph below the NRO data.

some other means, attempts were made to hybrid select transcripts located downstream of the initial PTC event(s) in region 8. An internal control was included in these experiments that would discount the possibility that a negative result (i.e., no selection of downstream transcripts) is due to nascent transcript degradation during the selection procedure. The internal control is provided by the hybrid selection of the 5′ end of the second intron (IVS2) of the β-globin gene, using a selection probe complementary to transcripts of the adjacent second exon (see diagrams in Figures 5A and 5B).

NRO hybrid selection analysis of βΔ5–7 transcripts, using exon 2 (Bio β2) and Bio β4 selection probes, is shown in Figure 5A. As in the previous analysis of βΔ5–7 transcripts, using the Bio β4 selection probe (see Figure 3A), strong hybridization signals are detected over probes 3, 4, and 8 with low level signals over probes 9 and 10, confirming that PTC occurs within region 8. The hybridization signals over the second exon and second intron probes, labeled 2 and IVS2, respectively, show that the control selection experiment has worked.

Selection of βΔ5–7 NRO transcripts with Bio β8 results in weak hybridization signals over probes 4, 8, and 9, with no signal detected over probe 10 (see Figure 5B; the lower signal over probe 8 reflects the variable recovery of RNA complementary to the selection probe). The hybridization signals over the exon 2 and IVS2 probes indicate that the low 9–10 signal is not due to RNA degradation. The observation that downstream transcripts are not retrieved using the Bio β8 probe points to a reiterative cleavage mechanism operating within the 8–10 termination region. Presumably, the hybridization signals detected over probes 9–10 by standard NRO analysis (see Figures 1 and 2B) represent short transcripts resulting from reiterative cleavage events in regions 8, 9, and 10.

To provide confirmation of this result, a further control experiment was carried out. An expression plasmid labeled βΔ5–7* in which regions 9–10 of the βΔ5–7 expression plasmid were replaced by regions 6 and 7 of the β-globin 3′ flanking region was constructed. As stated previously, regions 6–7 were chosen as they do not

support transcription termination or PTC activity. In NRO analysis of HeLa cells transiently transfected with βΔ5–7*, shown in Figure 5C, strong hybridization signals are detected over all probes within the β-globin gene and the 3′ flanking region. Background hybridization signals are detected over the plasmid probes A, B, and U3, indicating that the 3′ flanking region mediates full transcription termination. The corrected hybridization signals from this experiment are displayed in the graph in Figure 5D. Hybrid selection analysis of NRO-labeled βΔ5–7* transcripts using the Bio β4 probe, shown in Figure 5D, confirms that as with βΔ5–7 (see Figure 5A), selected transcripts do not extend beyond region 8 into the downstream probes. The corrected hybridization signals from this experiment are displayed alongside those from the βΔ5–7* NRO experiment in the graph beneath the NRO data. To examine whether the downstream region 6–7 transcripts could be retrieved, hybrid selection NRO analysis of βΔ5–7* transcripts was carried out using the Bio β8 probe as shown in Figure 5E. Strong hybridization signals are detected over probes 8, 6, and 7, indicating that the downstream region 6–7 transcripts have been successfully retrieved. The corrected hybridization signals from this experiment are displayed alongside those from the βΔ5–7* NRO experiment in the graph beneath the NRO data.

These experiments show that in the wild-type β-globin termination region, where the region 8 PTC element is followed by further sequences that can support PTC (regions 9–10), PTC occurs at all sequences. However, when region 8 is followed by sequence that does not support PTC (regions 6–7), then PTC is restricted to region 8. These results indicate that PTC is most likely a reiterative process that is based on sequence recognition.

Transcription Termination of the Human ϵ-Globin Gene

Next, the analysis of transcription termination of the β-globin gene was extended to that of the ϵ-globin gene. It has previously been shown by NRO analysis of ϵwt plasmid (previously HIVϵ; Dye and Proudfoot, 1999), consisting of the human ϵ-globin gene driven by the HIV LTR, that the first 1.65 kb of the ϵ-globin 3′ flanking region is sufficient to mediate pol II transcription termination (upper data panel, Figure 6A). The profile of transcription termination is different for this gene. Unlike β-globin, which abruptly terminates transcription at the end of the 3′ flanking region, ϵ-globin terminates transcription more gradually throughout the 1.65 kb of 3′ flanking region. To confirm that transcriptional termination of the ϵ-globin gene depends on the presence of a functional poly(A) site, an ϵ-globin construct (labeled ϵp(A)mut) was made. Inactivation of the poly(A) site was confirmed by RNase protection mapping of ϵp(A)mut transcripts as shown in Figure 6C. 3′ end processing at the poly(A) site gives a 150 nt band for ϵwt (lane 1), while ϵp(A)mut (lane 2) gives no band confirming that the poly(A) site is inactivated. In NRO analysis of ϵp(A)mut (lower data panel, Figure 6A), increased hybridization signals, as compared to ϵwt NRO analysis, are detected by probes A, B, and U3, confirming that inactivation of the ϵ-globin poly(A) site abolishes transcription termination.

Hybrid selection NRO analysis was then carried out on ϵwt transcripts using the Bio ϵ3 probe (Figure 6B; Dye and Proudfoot, 1999) and shows that uncleaved nascent transcripts extend throughout the 3′ flanking region. However, as compared to the unselected NRO data, signals over probes 7 to 9 are much lower and gradually reduce to near background levels. Note that the hybridization signal over probe 6 is artificially high due to cross hybridization of transcripts from region 4 of the ϵ-globin 3′ flanking region to probe 6. This is demonstrated by hybrid selection data obtained for NRO transcripts from an ϵ-globin plasmid with region 4 deleted (ϵΔ4, see middle panel of Figure 6B). In this analysis, the probe 6 hybridization signal more accurately reflects its true level relative to the rest of the 3′ flanking region probes and confirms that there is a gradual reduction of hybridization signals through the ϵ-globin 3′ flanking region.

To examine whether the selected transcript length is controlled by 3′ end processing at the poly(A) site or by sequences in the 3′ flanking region, ϵp(A)mut transcripts were also analyzed by NRO hybrid selection analysis using the Bio ϵ3 probe. Comparison of the selected ϵwt and ϵp(A) mut transcripts shows that the length of selected transcripts is unaffected by mutation of the ϵ-globin poly(A) site, suggesting that they are cleaved in a poly(A) site-independent manner by PTC, as shown in the β-globin system. A major difference between the ϵ- and β-globin genes is the greater length of the selected ϵ-globin transcripts. This observation indicates that there are no strong PTC elements, such as region 8 of the β-globin 3′ flanking region, in the ϵ-globin 3′ flanking region. Rather, it appears that transcript cleavage at weak PTC elements located throughout the 3′ flanking region leads to a gradual decrease in polymerase density. If there are such elements within the ϵ-globin 3′ flanking region, then removal of these sequences should have an effect on transcription termination.

To test the dependence of pol II transcription termination on sequences in the ϵ-globin 3′ flanking region, three deletion constructs termed ϵΔ4–9, ϵΔ4–6, and ϵΔ7–9 were made (see diagram in Figure 6D). In NRO analysis of ϵΔ4–9, shown in Figure 6D, strong hybridization signals are detected over probes A, B, and U3, confirming that the 3′ flanking region plays an important role in transcription termination. In more detailed analysis of the 3′ flanking region, by NRO analysis of ϵΔ4–6 and ϵΔ7–9, strong hybridization signals over A, B, and U3 are also detected, indicating that both the 5′ and 3′ halves of the 3′ flanking region are required for efficient transcription termination. The possibility that the 3′ flanking region deletions were affecting mRNA processing was tested by RNase protection analysis of steady-state RNA. No effect on 3′ end processing was detected (data not shown). These results confirm that sequences in the ϵ-globin 3′ flanking region have a direct involvement in pol II transcription termination.

Discussion

A Two Step Pol II Transcription Termination Model

Analysis of human β- and ϵ-globin gene transcripts demonstrates that although these genes are similar, they manifest distinct arrangements of termination signals. In the β-globin gene, transcription termination is mediated by strong termination signals which extend from 0.9 to 1.6 kb downstream of the poly(A) site. In contrast,

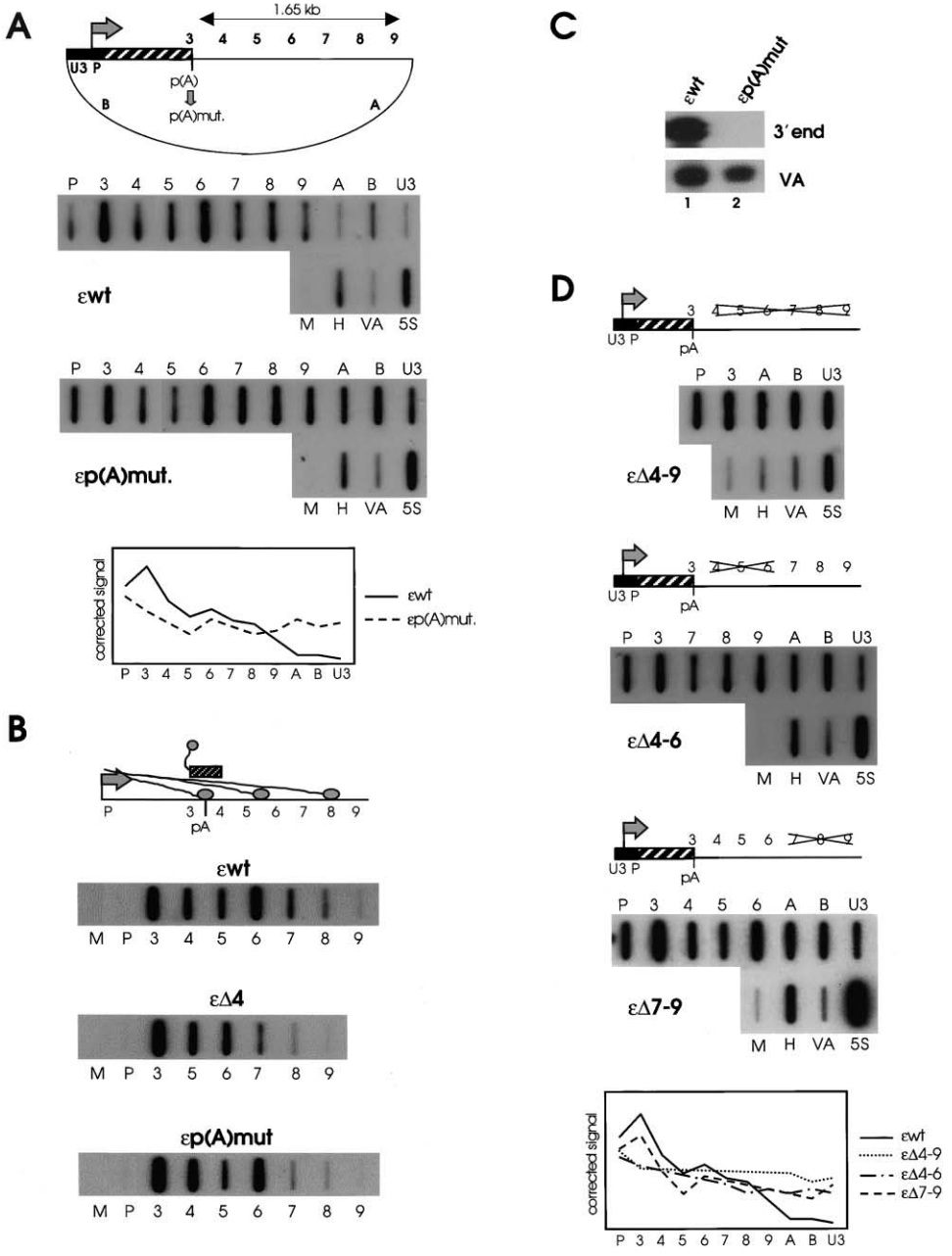

Figure 6. Transcription Termination Sequences in the ϵ-Globin 3′ Flanking Region

(A) NRO analysis of ϵwt and ϵp(A)mut plasmids. Composite diagram (not drawn to scale) of the ϵwt and ϵp(A)mut plasmid. Corrected hybridization signals are shown in the graph below the NRO data.

(B) Hybrid selection NRO analysis of ϵwt., ϵΔ4, and ϵp(A)mut transcripts. In the diagram, the position of the Bio ϵ3 selection probe with respect to the ϵ-globin 3′ flanking region is shown.

(C) RNase protection analysis of ϵwt. and ϵp(A)mut transcripts. The VA signal derives from the cotransfected VAI plasmid and controls for transfection efficiency and RNA recovery.

(D) NRO analysis of ϵΔ4–9, ϵΔ4–6, and ϵΔ7–9 plasmids. Corrected hybridization signals are shown in the graph below the NRO data.

it appears that transcription termination of the ϵ-globin gene is mediated by more diffuse signals, located throughout the 3′ flanking region.

Hybrid selection NRO analysis of transcripts spanning the "strong" termination sequences in the β-globin 3′ flanking region enables a dissection of the termination process. The data favors a model which predicts that pol II transcription termination occurs in two steps. In step 1, the nascent transcript is cleaved in a sequence-dependent manner by PTC (Figure 7A). Note that as pol II transcription termination is dependent on cotranscriptional 3′ end processing and in vitro experiments indicate a direct involvement of the carboxy-terminal domain (CTD) of pol II in this process (Hirose and Manley,

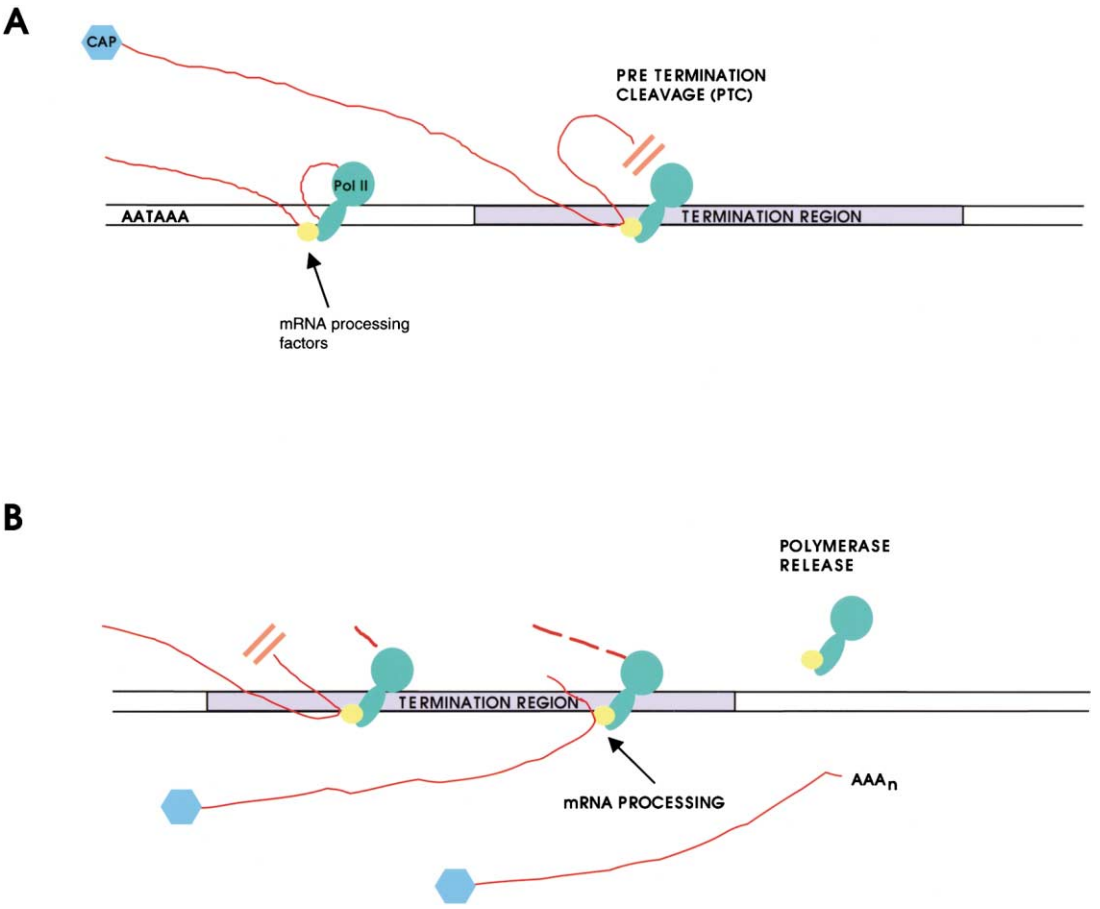

Figure 7. A Two Step Model for Pol II Transcription Termination

(A) Step 1: pretermination cleavage. In the diagram, the parallel black lines indicate the DNA template and AATAAA denotes the poly(A) signal. The red lines indicate the nascent RNA transcript and the blue hexagon attached to the transcript represents the mRNA cap structure. The thick parallel orange bars represent pretermination cleavage of the nascent transcript.

(B) Step 2: polymerase release. In this diagram, the thick broken line represents reiteratively cleaved transcripts. AAAn denotes the poly(A) tail.

1998), it is predicted that an indirect interaction of the transcript with the CTD may continue after PTC in order for cleavage/polyadenylation of the pre mRNA to take place. These proposed interactions may have a role in mediating the second step of the termination process, polymerase release, which occurs downstream of PTC and results in disengagement of pol II from the DNA template. Indeed these studies show that PTC operates independently of mRNA 3′ end processing while polymerase release is entirely dependent upon it. A likely scenario is that following PTC, mRNA 3′ end processing effectively triggers polymerase release (Figure 7B).

A spatial separation of pol II transcription termination events was suggested in a study of the *Drosophila hsp26* gene using in vivo UV crosslinking and $KMnO_4$ hyperreactivity site mapping (Giardina and Lis, 1993). Differences between top and bottom strand hyperreactivity on the *hsp26* 3′ flanking region DNA template indicated that the final 330 bp of the *hsp26* primary transcript was made by an altered, possibly less processive pol II complex. A precedent for the arrangement of termination signals in the human β-globin gene is provided by studies of *Xenopus laevis* ribosomal 40S precursor transcripts (Labhart and Reeder, 1986). Separate transcript and polymerase release sites, located downstream of the processing site that marks the 3′ terminus of the precursor 40S transcript, have been described. The authors suggest that transcript release might be mediated by the intrinsic transcript cleavage activity associated with pol I (Reeder and Lang, 1994).

Detailed analysis of the mouse β^{maj}-globin gene has revealed the presence of multiple poly(A) site-dependent termination signals in the 3′ flanking region (Tantravahi et al., 1993). These termination signals, the smallest of which is 69 bp, are believed to act in concert to mediate pol II transcription termination. It is not known if these sequences mediate PTC, as hybrid selection NRO analysis of the mouse β^{maj}-globin termination region has not been described. However, although no consensus sequence has been identified, the mouse termination sequences are AT rich. This characteristic is shared by the human β-globin 8–10 termination region, which contains AT rich tracts that might be important for PTC. Considering the above, it appears that there may be similarities between the mouse β^{maj}-globin and human β-globin transcription termination mechanisms.

A major difference, however, is that the efficiency of the mouse β^{maj}-globin termination elements is dependent on their distance from the poly(A) site whereas in the human β-globin gene, this does not appear to be the case (see Figure 2B).

Mechanism of PTC and Its Role in Polymerase Release

It seems plausible that PTC is brought about by a cotranscriptional RNA processing activity that targets 3′ flanking region sequences. The regulation of mRNA stability plays an important role in the control of gene expression (Mitchell and Tollervey, 2000). Although RNA degradation is thought of as a posttranscriptional event, a recent study in yeast has shown that a regulated pre-mRNA degradation pathway actually competes with the splicing machinery for pre-mRNA transcripts (Bousquet-Antonelli et al., 2000), indicating that it can occur very soon after transcription. It is possible that PTC is part of a general mechanism for the targeting and degradation of noncoding transcripts which, in conjunction with RNA processing, can stimulate pol II transcription termination.

Endonucleolytic cleavage of nascent transcripts mediated by RNA degradation machinery has not previously been described, presumably due to the limited number of in vivo nascent transcription studies. However, intrinsic transcript cleavage activities associated with all three classes of eukaryotic polymerase are well documented (Platt, 1998; Uptain et al., 1997). A number of in vitro studies suggest that the intrinsic transcript cleavage activity of pol II, which is enhanced by elongation factor SII, is used to overcome blocks to transcription elongation (Wind and Reines, 2000). Although no role for this activity in pol II transcription termination has been described, a connection between the pol III intrinsic transcript cleavage activity and transcription termination has been demonstrated (Chédin et al., 1998). Intrinsic transcript cleavage might alter the conformation of elongating pol II, making it less processive or more receptive to polymerase release signals. Additionally, intrinsic transcript cleavage and consequent exposure of an unprotected 5′ end on the nascent transcript might trigger RNA degradation.

Experimental Procedures

PCR Primers

A list of oligonucleotide sequences used as PCR primers is given in Supplementary Material (http://www.cell.com/cgi/content/full/105/5/669/DC1).

Plasmid Constructions

All DNA constructs were made by restriction digestion or long-range PCR using *Pfu* polymerase. Sequence coordinates refer to the GenBank sequence (accession number U01317). Construction of the βwt and βp(A)mut plasmids (formerly labeled HIVβ and HIVβp(A)mut) has been described previously (Dye and Proudfoot, 1999). The following constructs were made by PCR amplification of βwt using the oligonucleotide pairs shown in brackets: βΔ5–7 (3′B4/5′B8); βΔ5–8 (3′B4/5′B9); βΔ5–10 (3′B4/B10); βΔ8–10 (3′B7/B10); βΔ5–9 (3′B4/5′B10). βΔ5–7p(A)mut was made by PCR amplification of βp(A)mut using 3′B4 and 5′B8. The SV40 control plasmid, labeled pβeΔ5–7, was made by PCR amplification of pβe (Proudfoot et al., 1992) using oligos 3′B4 and 5′B8. βΔ5–7* was made by blunt end ligation of DNA fragment 8-6-7 (see below) to the linear βΔ5–10 product resulting from PCR amplification of βwt using oligo pair 3′B4/B10. The 8-6-7 fragment was made by blunt end ligation of region 6–7, and region 8 PCR products generated by amplification of βwt using the oligo pairs 5′B6/3′B7 and 5′B8/B9, respectively. Following ligation, the desired 8-6-7 fragment was PCR amplified from the ligation mix using the oligo pair 5′B8/3′B7. β8 was made by blunt end ligation of the region 8 fragment into the linear βΔ5–10 PCR product. βΔ5–9* was made by blunt end ligation of the region 6–7 fragment (see above) to the linear βΔ5–9 product resulting from PCR amplification of βΔ5–9 using oligo pair 3′B10/B10. εwt and εp(A)mut (formerly HIVε and HIVεp(A)mut) have been described previously (Dye and Proudfoot, 1999). εΔ4 and εΔ4–6 were made by PCR amplification of εwt using oligo pairs 3′E3b/5′E5 and 3′E3b/5′E7, respectively. εΔ4–9 was made by restriction digestion of εwt using AvrII (coordinate 21320) and XbaI, which cuts within the pUC polylinker downstream of the 3′ flanking region sequences, the large digestion product was blunt ended then religated. εΔ7–9 was made by restriction digestion of εwt at the SnaBI site (coordinate 21833) and the pUC polylinker XbaI site, the large digestion product was blunt ended then religated. The Tat and VA plasmids have been described previously (Dye and Proudfoot, 1999).

NRO probes for the β-and ε-globin genes and A, B, H, 5S, P, U3, VA have been described previously (Dye and Proudfoot, 1999). The β-globin exon 2 and IVS2 NRO probes were made by insertion of PCR products resulting from PCR amplification of βwt with oligo pairs 5′B2/3′B2 and 5′IVS2/3′IVS2, respectively, into M13mp18.

The Bio β3, β4, and ε3 selection probes have been described previously (Dye and Proudfoot, 1999). The Bio β8 selection probe was made by insertion of the βwt (5′B8/3′B8) PCR product into pGEM3. The Bio β2 selection probe was made by insertion of the βwt (5′B2/3′B2) PCR product into pGEM4. The β8/9 riboprobe was made by insertion of the βwt (5′B8/3′B9) into pGEM4. The βΔ5–7 3′ flanking region control transcript was made by insertion of the βΔ5–7p(A)mut (5′B4/3′B10) PCR product into pGEM3. β-globin wt and p(A)mut 3′ riboprobes were made by restriction digestion of βΔ5–7 and βΔ5–7p(A)mut constructs with BstXI (coordinate 63511) and AvrII (coordinate 64615). The 63511–64615 fragments were inserted into pGEM4.

Transfection Procedure

Transient transfection of HeLa cells was performed as described previously (Dye and Proudfoot, 1999).

RT-PCR Analysis

cDNA was generated using SuperScript II RT (Gibco BRL). cDNAs were amplified (25 cycles) using Taq polymerase under standard conditions. The oligo names used in the text refer to the following oligonucleotides: a is B3, b is B8, c is B9, d is 3′B9, e is 3′B10.

RNase Protection

Riboprobe preparation and RNase protection analysis were performed as described previously (Ashe et al., 1997).

NRO, Hybrid Selection NRO Analysis, and Biotin Labeling of RNA Selection Probes

Detailed protocols for these techniques are described in Dye and Proudfoot (1999).

RNase Protection Mapping of Hybrid-Selected NRO Transcripts

Preparation of labeled nascent RNA and hybridization to biotinylated probe (500 ng) and cold riboprobe (500 ng) was performed as described previously (Dye and Proudfoot, 1999). After the initial magnetic selection, the bead/RNA pellet was washed, in 300 μl of RNase protection buffer, and magnetically selected twice to remove nonhybridized transcripts. The bead/RNA pellet was resuspended in 300 μl of RNase protection buffer, RNase A (40 μg/ml) and RNase T1 (1000 U/ml), and incubated at 18°C for 90 min. (Endonucleolytic cleavage of selected transcripts in an unprotected gap, between the selection probe and cold riboprobe, enables the separation of protected transcripts from the selection probe/bead conjugate.) Following RNase digestion, the probe/bead conjugate was precipitated by magnetic selection. The supernatant fraction was removed to a fresh tube and 30 μl of Proteinase K mix (3.3 mg/ml proteinase K. 7.7% SDS) was added. Following incubation at 37°C for 30 min,

RNA was purified by phenol chloroform extraction and ethanol precipitation, then fractionated by electrophoresis and visualized by autoradiography.

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