Nucleic Acids Research, 1994, Vol. 22, No. 14 2811–2816

Polyadenylation and transcription termination in gene constructs containing multiple tandem polyadenylation signals

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Received March 21, 1994; Revised and Accepted June 13, 1994

ABSTRACT

The processes of pre-mRNA 3′-end cleavage and polyadenylation have been closely linked to transcription termination by RNA polymerase II. We have studied the relationship between polyadenylation and transcription termination in gene constructs containing tandem poly(A) signals, at least one of which is the inefficient polyomavirus late poly(A) site. When identical tandem viral signals were separated by fewer than 400 bp, they competed for polyadenylation. The upstream site was always chosen preferentially, but relative site choice was influenced by the distance between the signals. All of these constructs showed the same low level of transcription termination as wild type polyomavirus, which contains a single late poly(A) site. When tandem poly(A) signals were not identical, a stronger downstream signal could outcompete a weaker upstream signal for polyadenylation without altering the efficiency of transcription termination characteristic for use of the upstream signal. Thus, if a weak polyoma virus late poly(A) signal (associated with inefficient transcription termination) preceded a strong rabbit β-globin signal (associated with efficient transcription termination), termination remained inefficient, but the distal signal was most often chosen for polyadenylation. These results are consistent with independent regulation of polyadenylation and transcription termination in this system and are discussed in light of current models for the dependence of transcription termination on a functional poly(A) site.

INTRODUCTION

For most eukaryotic RNA polymerase II transcription units, 3′ end formation involves an endonucleolytic cleavage and addition of 50–200 adenylic acid residues to the precursor (1, 2). In most genes, 3′ processing signals include the hexanucleotide 5′-AAUAAA-3′ (3–5) and a GT- or T-rich region about 7–43 bp downstream of it (6, 7). Proteins that specifically interact with the AAUAAA or the GT-rich region have already been identified (see 8 for review). It is known that other elements may also play a role in polyadenylation, but the mechanism by which these elements function is still not clear.

Transcription termination and polyadenylation are closely linked *in vivo* (9–12). RNA polymerase II transcription termination occurs hundreds or thousands of nucleotides downstream of polyadenylation sites (7, 13, 14). Although there have been a number of reports of specific sequences which may act as termination sequences (15–25), *in vivo*, transcription termination often depends on 3′-end processing (9–12, 26). Mutations in the conserved polyadenylation AAUAAA signal of the mouse major β-globin gene or the human globin gene, respectively (11, 12), or mutations in both the AAUAAA hexanucleotide and the downstream GT-rich region of the SV40 early polyadenylation site (9) can inhibit polyadenylation as well as transcription termination. Further, insertion of a strong poly(A) signal (such as the rabbit β-globin poly(A) site) upstream of a weak poly(A) signal (such as the polyomavirus late poly(A) site) can induce efficient transcription termination (10).

Two models have been proposed to account for the linkage between transcription termination and polyadenylation (9, 11, 14, 27), but little direct evidence for or against any model has been obtained. The first model postulates that termination is triggered by 3′ cleavage of the nascent transcript. Following cleavage, the newly formed, uncapped 5′ end would serve as the entry site for a termination factor, or a 5′-to-3′ exonuclease, which would chase the polymerase and eventually destabilize the transcription complex (9, 14, 27). The second model suggests that the transcription complex contains a processivity or antitermination factor, which is released at the polyadenylation signal (11).

We have studied the *in vivo* relationship between polyadenylation and transcription termination in gene constructs containing tandem poly(A) signals, at least one of which is the polyoma virus late polyadenylation signal, which is associated with both inefficient polyadenylation and transcription termination (28). Identical tandem signals competed in a distance-dependent fashion for polyadenylation, with the upstream site always chosen

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preferentially. When tandem poly(A) signals were not identical, a more efficient downstream signal outcompeted a weaker upstream one for polyadenylation. For all constructs, however, transcription termination efficiency was determined by the upstream signal. These results are consistent with independent regulation of polyadenylation and transcription termination and are discussed in the context of current models for the dependence of transcription termination on a functional poly(A) site.

MATERIALS AND METHODS

Materials

Restriction enzymes, DNA polymerase I Large Fragment (Klenow enzyme), T4 DNA ligase, T4 DNA polymerase, and T4 polynucleotide kinase were obtained from New England Biolabs, and used as suggested by the supplier. *E.coli* strains JM83, and UT481 were used to propagate plasmids by standard procedures (29, 30). SuperScript® reverse transcriptase was obtained from Bethesda Research Laboratories. Oligonucleotides were synthesized using a Milligen/Biosearch Cyclone DNA synthesizer. RNase T1/T2 for ribonuclease protection assays was from Bethesda Research Laboratories, or was prepared as described (31). Polymerase chain reaction analysis (PCR, 32) was performed using the GeneAmp® kit from Perkin Elmer Cetus. Sequencing was performed by the dideoxy method using the Sequenase® kit from United States Biochemical Corp. [α-^{32}P]-labeled deoxynucleoside and ribonucleoside triphosphates and [γ-^{32}P]ATP were obtained from New England Nuclear. All polyoma constructs were made using the strain 59RA (33, 34).

Cell culture techniques, transfections, and nuclei and RNA isolations

Mouse NIH3T3 cells were propagated, and transfections performed using a modification of the Chen and Okayama procedure (35) as described elsewhere (36, 37). At 44 h after transfection, cytoplasmic RNA was isolated using modifications of procedures as previously described by our laboratory (36, 38).

Cytoplasmic RNAs were analyzed by RNase T2 protection experiments as previously described (39). The riboprobe used for the double poly(A) site constructs pRUEY-4, pRUEY-5 and pRUEY-6 was made by inserting a 147 bp polyoma *Hin*cII−*Hgi*AI fragment spanning the late poly(A) site into the *Hin*cII site of plasmid pBS$^+$, followed by transcription with T7 RNA polymerase. Protected bands of 147 nt represent failure to use the upstream poly(A) signal, while bands of 78 nt represent poly(A) site cleavage at either polyoma site. The riboprobe used for constructs pRUEY-5 and pRUEY-10 in the experiment of Fig. 5 was made by inserting a *Bbv*I−*Bam*H1 fragment of pRUEY-10 into pBlueScript SKII, followed by transcription with T3 RNA polymerase. Protected bands of 184 nt represent use of the upstream site in pRUEY-5 and pRUEY-10. Use of the downstream site in pRUEY-5 produces a 448 nt band, and use of the downstream site in pRUEY-10 produces a 498 nt band.

Labeled riboprobes were annealed to 20−25 mg cytoplasmic RNA at 57°C for 15 to 20 h. The resulting hybrids were digested with T2 RNase or crude T1/T2 RNase at a final concentration of 60 U/ml at 37°C for 1.5 h. After phenol extraction and ethanol precipitation, the samples were dissolved in denaturing dye, heated to 92°C for 2 min, chilled on ice, and protected bands resolved on 6% polyacrylamide-urea sequencing gels.

PCR assays

PCR analysis was as described (40). Briefly, oligonucleotide 275 (5′-TATCACCGTACAGCCTTG) was made complementary to the late mRNA for VP1, and spans the junction site of the late leader to this exon. Oligonucleotide 278 (5′-CCTGACATTTT-CTATTTTAAG) binds to a reverse transcript of the immediate upstream region of the late leader mRNA. Cytoplasmic RNAs were isolated from transfected cells, and copied with SuperScript reverse transcriptase using oligonucleotide 275 as a primer at 42°C for 1 h in SuperScrip reverse transcription buffer. Taq polymerase and oligonucleotide 278 were then added and the mixtures cycled as follows. Samples were incubated at 94°C for 2 min, followed by 20 cycles of incubation at 92°C for 1.5 min (melting), 45°C for 1 min (annealing), and 72°C for 1.5 min (extension). Samples were then incubated at 72°C for 10 min, and brought to 4°C. Leaders were resolved by electrophoresis on 6% polyacrylamide/7M urea sequencing gels, and quantitation was with a Betagen Betascope Blot Analyzer.

Construction of mutants

Plasmid pRUEY-4 was constructed by inserting a *Hin*cII−*Rsa*I fragment spanning the polyoma late polyadenylation site of probe PP4 (41) into the *Hin*cII site of a plasmid (pRUEY-1) which contains the polyoma virus genome cloned into a modified pUC8 vector (41). For pRUEY-5, a slightly longer *Hin*cII−*Hae*III fragment from the same parent was inserted. For pRUEY-6, the inserted fragment was *Hin*cII to T4-blunted *Hgi*AI. Construct pPyβAA contains both the *Hin*cII−*Apa*I fragment of polyoma and the *Sal*I fragment of rabbit β-globin (10) cloned in a head-to-tail orientation into pBS$^+$. Construct pRUEY-10 was made by inserting the *Hin*cII fragment (containing both the polyoma and β-globin poly A signals) from clone pPyβAA, into the *Hin*cII site of pRUEY-1. Plasmid pR-βG was made by cloning the *Apa*I fragment containing the β-globin polyadenylation site of mutant ins5 (10) into the *Apa*I gap of pRUEY-1.

For the experiments shown in Figure 6, a series of related constructs were prepared. Plasmid p**A** was created by site directed mutagenisis and will be described in detail elsewhere. Briefly, the polyoma late polyoma's poly(A) signal was replaced with a strong, well-characterized synthetic poly(A) site (50), while preserving the viral early coding region and early poly(A) signal on the opposite DNA strand. Plasmid p**a-a** was made by inserting a fragment comprising 50 bp upstream to 150 bp downstream of the wild type polyoma late poly(A) signal into the *Hin*cII site of the wild type clone. This created a construct with the weak wild type signal 201 bp upstream of another weak wild type signal. Construct p**a-A** was similar, except the downstream poly(A) site was the synthetic one, and the spacing was 207 bp. Construct p**A-a** was similar, except the upstream poly(A) site was the synthetic one, and the spacing was 199 bp. Construct p**A-A** was similar, except both poly(A) signals were the synthetic ones, and the spacing was 205 bp.

RESULTS

Wild type polyoma virus contains a single weak late polyadenylation signal. Several constructs were made with duplicated polyadenylation signals, at least one of which was the polyoma late signal (Fig. 1). The spacing between tandem polyoma late poly(A) signals varied from 147 bp (construct pRUEY-6) to 390 bp (construct pRUEY-4). In pR-βG, the

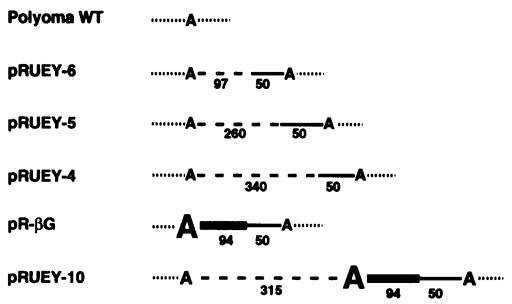

Figure 1. Some of the constructs used in this study. Only the poly(A) site region of each construct is depicted; all constructs are identical both upstream of the first poly(A) signal and downstream of the last poly(A) signal, and all are in pUC-based plasmids which contain the entire polyoma virus genome. The numbers represent the number of nucleotides either upstream or downstream of the AAUAAA signals. The thick hatched bar represents sequences from the downstream region of the rabbit β-globin poly(A) signal (10). The large A indicates the rabbit β-globin polyadenylation signal. The small A represents the polyoma late polyadenylation signal. WT, wild type strain 59RA (33).

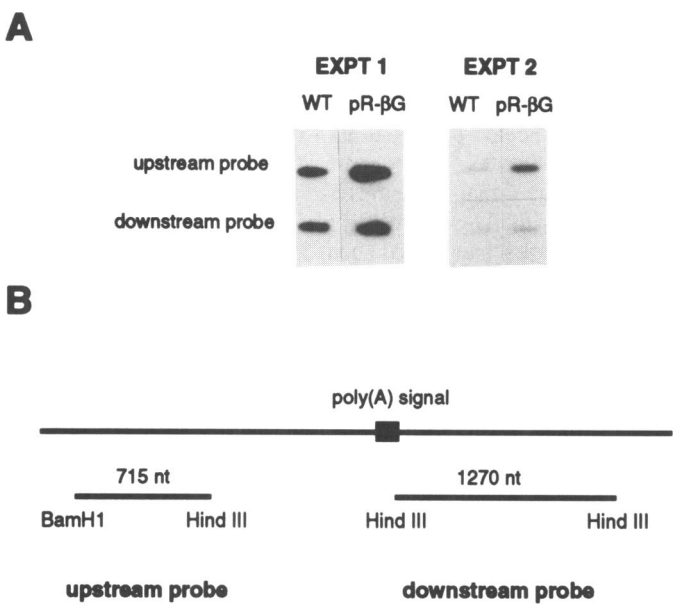

Figure 2. Analysis of transcription termination efficiency of wild type and construct pR-βG using nuclear run-on assays. (**A**) NIH 3T3 cells were transfected with wild type or pR-βG genomes as described in Materials and Methods, and nuclear run-on assays performed as described previously (38). Labeled RNA was annealed to immobilized single-stranded DNA probes specific for late-strand transcripts. (**B**) Schematic diagram of the upstream and downstream probes. The upstream and downstream probes were previously-described (probes E′ and C′, respectively; 38). Use of an immobilized probe spanning the polyadenylation signal region of the two viral constructs was not allowed since these constructs differed in sequence in this region.

upstream signal (the AAUAAA hexanucleotide and 94 downstream bases) derives from the rabbit β-globin gene. In construct pRUEY-10, this heterologous signal was placed 315 bp downstream of the polyoma late signal.

Construct pR-βG contains the genome of our parent laboratory strain 59RA, but is identical in its poly(A) region to mutant ins5, which has been shown to exhibit efficient late-strand

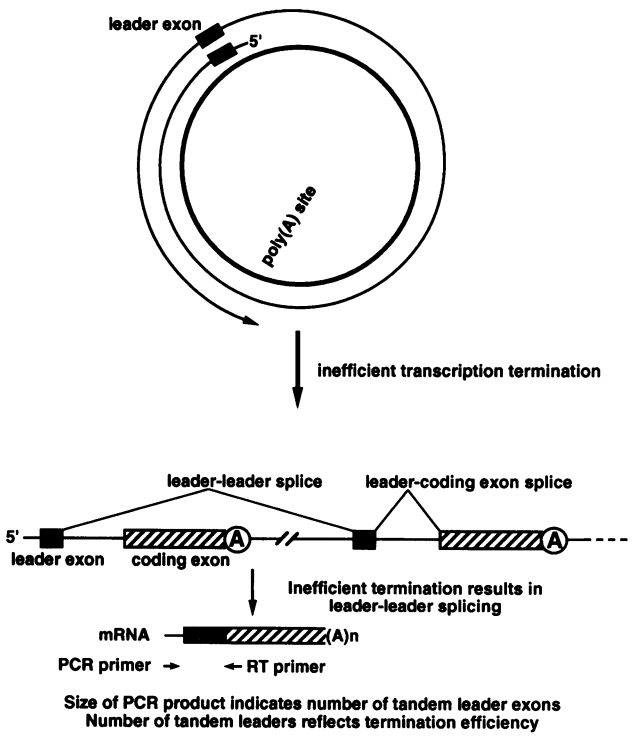

Figure 3. Strategy used to assay transcription termination by determining the number of tandem late leader exons on the 5′-ends of polyoma virus mRNAs. Due to inefficient termination by RNA polymerase II late viral transcripts are heterogeneous in length, with many representing multiple circuits of the viral genome by the polymerase. The example shown is for a transcript created by two passes through the viral late region. This transcript has two leader exons (shaded boxes) and two message body coding exons (hatched boxes). During pre-mRNA processing a leader−body splice joins the 3′-most non-coding late leader exon to a message coding body. In addition, leader−leader splicing removes a genome length intron, yielding a final message with two tandem leader units at its 5′-end. Inefficient termination is thus measured by leader multiplicity on mature messages. The reverse transcription and PCR methods used to assay termination have been described in detail (40). The inefficient polyoma late poly(A) signal is denoted by the circled A.

polyadenylation and transcription termination (10). In order to confirm that the rabbit β-globin poly(A) signal of pR-βG directs efficient transcription termination relative to our wild type construct, nuclear run-on assays were performed (Fig. 2). Labeled product RNAs were annealed to single-strand probes representing sequences both upstream and downstream of the polyadenylation signal regions of wild type and pR-βG. Quantitation was using a Betascope Blot Analyzer. Raw counts were corrected for U-content of the upstream and downstream probes, and relative termination efficiencies calculated from the ratios of the signals. In two independent experiments (Fig. 2A), the termination efficiency of wild type virus transcription between the two probes was 34% in each case. In both experiments, however, the termination efficiency for pR-βG was 63%.

As nuclear run-on assays are tedious and time-consuming, we have chosen to use a RT-PCR assay to determine transcription termination efficiency in the polyoma virus system (40). This assay (Figure 3) takes advantages of the unusual nature of polyoma late gene expression in which both transcription termination and polyadenylation are inefficient (42, 43). Late

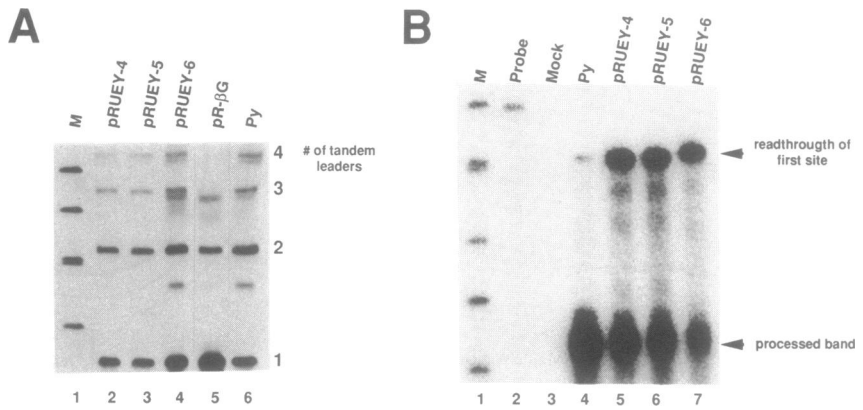

Figure 4. Analysis of poly(A) site choice and transcription termination in constructs with tandem poly(A) sites. (**A**) PCR assay results. The positions of bands representing one leader, two leader and three leader messages are indicated. The band at approximately the position of three leaders in the pR-βG lane (lane 5) is an artifact band not seen in other experiments (for example, see Fig. 5A, lane 3). (**B**) T2 RNase protection assay results using cytoplasmic RNA isolated after transfections. The assay was performed as described (31). Bottom bands (centered around 78 nt) represent the use of both upstream and downstream polyadenylation sites. The top bands (147 nt) represent messages not processed at the upstream sites, and therefore presumably processed at the downstream polyadenylation sites. Quantitation was using a Betagen Betascope Blot Analyzer. Results were corrected for probe length and U content, with the assumption that processing at the first site would yield only the smaller band, while processing at the downstream site would yield both bands. Py, polyoma.

transcription complexes can continue around the circular genome multiple times, in the process transcribing tandem copies of the viral late region. Such transcripts contain more than one copy of the noncoding late leader exon, and are processed by the sequential splicing of these leader exons to each other (41, 44; Fig. 3). Thus, late mRNAs contain variable numbers of tandem leaders at their 5'-ends, with the leader multiplicity being an indicator of low transcription termination efficiency.

In the first step of our RT-PCR assay (Fig. 3), an oligonucleotide spanning the splicing junction of the leader and the predominant message body exon is annealed to cytoplasmic RNA from transfected cells, and extended using reverse transcriptase. The second step of PCR amplification includes a 5'-end labeled oligonucleotide that binds just upstream of the late leader. The sizes of bands generated reveal the number of tandem leaders; the intensities indicate the fraction of messages with particular numbers of leader units. Using this method, it was determined that about 70% of RNA polymerase II molecules terminate each time around the polyoma genome (40). This is in good agreement with the predicted distribution based on direct measurements of late transcription termination using ^{3}H-uridine pulse labeling (43). The RT-PCR assay we use also gives the same quantitative results as RNAse protection assays for leader−leader splicing (data not shown).

RT-PCR termination results (Fig. 4A) clearly show that in constructs pRUEY-4, pRUEY-5 and pRUEY-6, leader to leader splicing occurs as often as for wild type. For each, the deduced termination efficiency was about 70%. These results confirm that transcription passes through the weak polyoma polyadenylation multiple times (Fig. 2 and 28), but also show that multiple signals do not appear to act additively to increase termination. Construct pR-βG, which contains the rabbit β-globin poly(A) signal first, showed significantly greater termination efficiency (>90%; Fig. 4A, lane 5 and Fig. 5A, lane 3). This is consistent with the results of Fig. 2 and with a previous report that the rabbit β-globin polyadenylation site can function as an efficient polyadenylation/termination site in the polyoma virus late region (10).

To test the relative use of both polyadenylation sites, we used a riboprobe spanning the polyoma late site. Protection of a fragment of 147 nt would reflect messages processed only at the downstream site of pRUEY-4, pRUEY-5 and pRUEY-6, while a shorter protected band (78 nt) would reflect polyadenylation events at both sites. In order to calculate polyadenylation site efficiency, the signal intensities had to be corrected for protected band lengths and U-contents. In pRUEY-6 (Fig. 4B, lane 7, and Table 1), the first site is used about 55% of the time. If the second polyadenylation site is positioned further downstream as in constructs pRUEY-5 and pRUEY-4, the readthrough band intensity decreased, indicating that 75% of late messages used the upstream polyadenylation site for pRUEY-5, and 87% used the first site in pRUEY-4. In all cases, overall levels of cytoplasmic RNA recovered were comparable (data not shown), ruling out significant influences of multiple poly(A) sites on RNA transport or degradation. The conclusion from these experiments is that spacing between sites contributes to poly(A) site choice in this system, with the upstream site always preferred. In multiple repeats of this experiment, essentially the same numbers were consistently obtained.

To test whether polyadenylation site strength can also influence poly(A) site selection in a system containing multiple polyadenylation sites we analyzed pRUEY-10 (Fig. 5B, lane 1). If polyadenylation site choice in constructs containing multiple signal results from competition for polyadenylation, then placing the efficient β-globin polyadenylation site upstream or downstream of a weak polyoma polyadenylation site should affect the relative use of the polyoma polyadenylation site. Results from analysis of cytoplasmic mRNA from pRUEY-10 and pR-βG transfection proved this. When the rabbit β-globin polyadenylation signal was placed upstream of the polyoma late signal (pR-βG), termination was efficient (Fig. 5A, lane 2 and Fig. 6A, lane 4) and polyadenylation was almost exclusively from the upstream globin site (data not shown, Table 1 and Fig. 6B, lane 4). This is consistent with previous results using a similar construct (28). When the β-globin polyadenylation signal was placed 315 nt downstream of the polyoma late signal (pRUEY-10), it competed effectively with the viral signal for polyadenylation, with 63% of messages processed at the downstream, β-globin site. This compares to only 25%

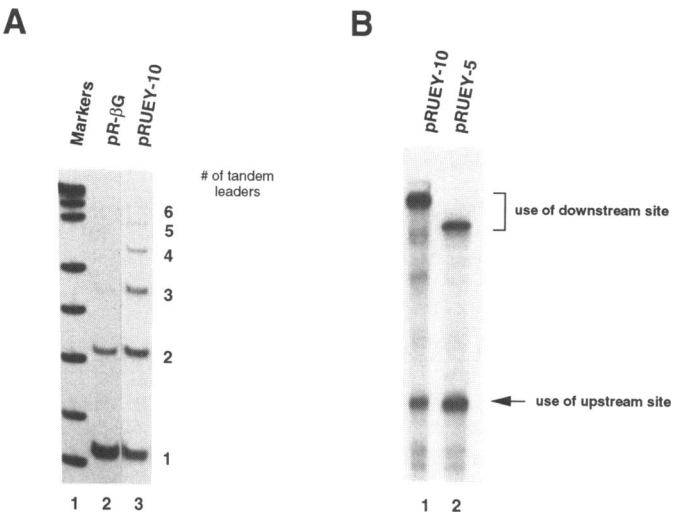

Figure 5. Analysis of poly(A) site choice and transcription termination in constructs with tandem poly(A) sites of different strength and spacing. (**A**) RT-PCR assay results of pR-βG and pRUEY-10. The positions of bands representing messages with one or more leaders are indicated. Lane 1, pUC18 *MspI* markers. (**B**) T2 RNase protection assay results using cytoplasmic RNA isolated after transfections with pRUEY-10 and pRUEY-5. Bottom bands represent the use of upstream polyadenylation sites. The top bands represent messages processed at the downstream polyadenylation sites. Lane 1, pRUEY-10. Lane 2, pRUEY-5.

Table 1. Relative poly(A) site use

Construct	Use of first site (%)	Use of second site (%)	Ratio site 1/site 2 use
pRUEY-6	55	45	1.2
pRUEY-5	75	25	3.0
pRUEY-4	87	13	6.7
pRUEY-10	37	63	0.59
pR-βG	>95	<5	>20

processing at the downstream site in pRUEY-5, which has a similar spacing of polyadenylation signals (Table 1 and compare Fig. 5B, lanes 1 and 2). However, transcription termination efficiency in pRUEY-10 remained at the same low level observed for wild type polyoma (Fig. 5A, lane 3). This was in spite of the fact that construct pRUEY-10 actually contains three, not two polyadenylation signals, and one of these signals is the rabbit β-globin one.

In order to demonstrate that another efficient polyadenylation signal could yield similar results, a series of additional constructs were made and tested (Fig. 6). Construct '**A**' (lane 6) contained a strong synthetic poly(A) signal (50) in place of the weak polyoma late signal (construct '**a**', lane 5). Constructs '**a-a**', '**A-A**', '**a-A**' and '**A-a**' (lanes 1–4) each contained two poly(A) sites of equal or different strengths separated by 200 bp. When analyzed by our late leader RT-PCR assay, all constructs with the synthetic poly(A) signal ('**A**') in the upstream position show more efficient transcription termination, while those with the polyoma signal ('**a**') upstream show poorer termination (Fig. 6A). This conclusion is evident from inspection of the drop-off in multiple late-leader band intensities in lanes 2 and 4 relative to lanes 1 and 3. As was observed above for pRUEY-10, the stronger poly(A) signal is always preferred, regardless of position

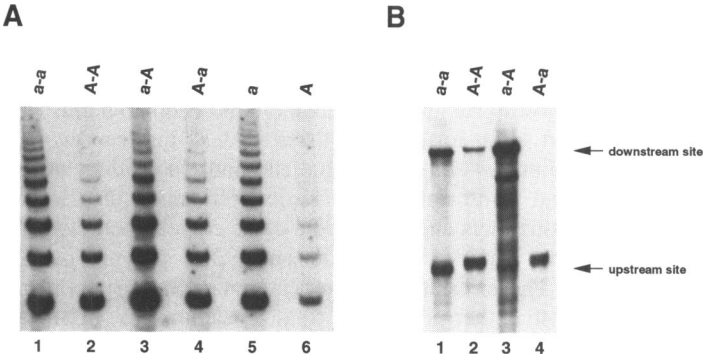

Figure 6. Analysis of poly(A) site choice and transcription termination in constructs with tandem poly(A) sites of different strengths, but with the same spacing. (**A**) RT-PCR assay results of wild type ('**a**', lane 5), a construct with a synthetic poly(A) site ('**A**', lane 6), and constructs containing combinations of the two (**a-a**, **A-A**, **a-A** and **A-a**), with about 200 bp spacing between them (lanes 1–4). (**B**) T2 RNase protection assay results using cytoplasmic RNA isolated after transfections with constructs **a-a**, **A-A**, **a-A** and **A-a**. Bottom bands represent the use of upstream polyadenylation sites. The top bands represent messages processed at the downstream polyadenylation sites. For each construct an individual riboprobe was prepared using PCR of the plasmid constructs, followed by subcloning into pBlueScript and transcription with T7 RNA polymerase. For each construct, use of the upstream poly(A) site is reflected by a band of about 220 bp, while use of the downstream site is reflected by a band of about 410 bp.

(Fig. 6B). Thus, for construct **a-a** (lane 1) the upstream site is used 65% of the time, for construct **A-A** (lane 2) the upstream site is used 84% of the time, for construct **a-A** (lane 3) the first site is used only 34% of the time, and for **A-a** (lane 4) the first site is used 98% of the time.

DISCUSSION

Results from a number of laboratories have shown that when two polyadenylation sites are duplicated and separated by relatively short distances, the upstream polyadenylation site is used preferentially (19, 28, 45–48). However, one study showed that for duplicated poly(A) sites, a stronger downstream one could outcompete a weaker upstream one when less than 400 bp away (19). In those instances where RNA levels were measured, there was no significant change in mRNA amounts produced by constructs with multiple signals (28, 45, 47). Spacing is also important: increasing the distance between two identical signals favored the use of the 5′-most site (45, 49). For example, Denome and Cole (45) made a number of constructs with multiple polyadenylation signals, with variable spacing between them. These authors found that all polyadenylation signals could be used, but that increasing the distance between two identical signals favored the use of the 5′-most site. Similarly in our system, when tandem polyadenylation signals were spaced further apart, the downstream signal appeared to have less influence on polyadenylation at the first site. When sites were separated by greater than 1.4 kb, the downstream site was almost never used (data not shown).

Two models have been proposed for transcription termination by RNA polymerase II in genes containing poly(A) signals. The first postulates that termination is triggered by 3′ cleavage of the nascent transcript. Following cleavage, the newly formed, uncapped 5′ end would serve as the entry site for a termination factor, or a 5′-3′ exonuclease, which would chase the polymerase

and eventually destabilize the transcription complex (9, 14, 27). The second model suggests that the transcription complex contains a processivity or antitermination factor, which is released at the polyadenylation signal (11). To date there has been no published direct support so far for either of these models. In recent work, Edwalds-Gilbert *et al.* (26) studied both polyadenylation efficiency and transcription termination efficiency in a large number of constructs and observed a close correlation between 3′ processing complex formation and RNA polymerase II termination. These results, however, do not strongly support or argue against either model for the relationship between polyadenylation and transcription termination.

The data presented here are not compatible with the first model described above. If cleavage triggered termination, then construct pRUEY-10, which contains the efficient rabbit β-globin polyadenylation signal, should produce few multigenomic transcripts that are processed to mRNAs with multiple late leader exons. However, this construct showed about 70% termination efficiency (the same as wild type polyoma virus), in contrast to constructs having the β-globin signal proximal to the viral one (greater than 90% termination). The same results were obtained when the rabbit β-globin polyadenylation signal was replaced with a previously characterized synthetic poly(A) signal (50; and see Fig. 6). Repeated experiments consistently yielded similar results. Although even more constructs need to be tested in future work, the data presented here are consistent with a model in which the processivity of elongating RNA polymerase II is in some way altered by recognition of the first polyadenylation signal encountered during transcription, regardless of whether that signal is the one used for polyadenylation. Whether an alteration in polymerase II processivity results from a *cis*-acting DNA signal, an RNA signal, or the acquisition or loss of a protein factor from the elongation complex awaits further experimentation.

ACKNOWLEDGEMENTS

We thank N.Acheson for providing mutant ins5, and N.Barrett, D.Batt, E.Carmichael, Z.Liu and L.Rapp for helpful comments on this manuscript. This work was supported by grant CA45382 from the National Cancer Institute.

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