Nucleic Acids Research, Vol. 19, No. 13 3593–3599

Termination of transcription in an *'in vitro'* system is dependent on a polyadenylation sequence

Vicente J.Miralles
Departament de Bioquìmica i Biologìa Molecular, Facultat de Farmàcia, Universitat de Valèncìa, Avgda. Blasco Ibáñez, 13, 46010 Valencia, Spain

Received March 27, 1991; Revised and Accepted May 29, 1991

ABSTRACT

Using HeLa cell nuclear extract as a source of the different transcription and polyadenylation factors and reverse transcription to analyze the levels of RNA 5′ and 3′ to the cleavage-polyadenylation site, an *in vitro* assay has been established to study polyadenylation coupled to transcription directed by different adenovirus promoters. The levels of transcription 5′ and 3′ to the cleavage site in the L3 polyadenylation region are practically the same as described previously, however, the level of transcription 3′ to the cleavage site in the SV40 early polyadenylation region decreases immediately after the cleavage site indicating a termination of the transcription.

INTRODUCTION

The 5′ and 3′ ends of the majority of eukaryotic mRNAs are modified after transcription initiation. The 5′ end of the mRNA is 'capped' by the addition of a guanosin nucleotide. Capping occurs almost immediately after the initiation of transcription and among other functions [1,2] protects the elongating RNA chain from degradation [3,4]. Formation of the 3′ ends of nonhistone eukaryotic mRNAs involves the post-transcriptional addition of a poly(A) tract of approximately 200–300 nucleotides in length. How RNA polymerase II terminates transcription is still not well understood. In all eukaryotic non-histone mRNA studied thus far, RNA polymerase II transcribes across the polyadenylation site(s) and terminates hundreds or even thousands of nucleotides downstream from the poly(A) sites [5]. Termination seems to occur at multiple sites as observed by a gradual reduction in transcription downstream of the poly(A) region (for review see 6, 7).

Although specific DNA sequences have been identified as termination signals for different genes [8–14], there is strong evidence to support the idea that a functional polyadenylation site, besides these possible termination sequences, is required for transcriptional termination. The polyadenylation site of most eukaryotic genes contains two and sometimes three conserved sequence elements. The highly conserved and ubiquitous AAUAAA is located 10 to 30 nucleotides upstream of the polyadenylation site. The cleavage site is a dinucleotide which shows a clear preference for the sequence CA . Finally, some genes have a third element, downstream of the poly(A) site which is less conserved than the other two and consists of a GU-rich or U-rich sequence. The AAUAAA sequence appears to be essential for cleavage and polyadenylation in vivo as well as in vitro, and there is also evidence indicating that the downstream sequences play an important role in the polyadenylation event (for review see 15).

It has also been reported that the polyadenylation sequence elements are necessary for termination of transcription. Whitelaw and Proudfoot [16] and Logan et al. [17] have shown that a single base change in the AATAAA sequence of the human-globin gene and the mouse major β-globin gene, respectively, not only decreases the polyadenylation process, but also increases transcription downstream of the polyadenylation site. Further, Lanoix and Acheson [18] found that insertion of a strong polyadenylation site, such as the rabbit β-globin poly(A) site, upstream of a weak polyomavirus late poly(A) site, increases the overall efficiency of the polyadenylation of polyoma late transcript. Additionally, in this case the termination of transcription by RNA polymerase II is also increased when compared with wild type virus. Experiments carried out by Connelly and Manley [19], in which transient expression assays were used to analyze the RNA produced from DNA constructs composed of the adenovirus major late promoter and the SV40 early polyadenylation site demonstrated that both the AATAAA hexanucleotide and the GT-rich region were required for transcription termination by RNA polymerase II.

An *in vitro* assay has been used here which permits the study of polyadenylation coupled to transcription. In this system it was possible to observe termination of transcription when the SV40 early polyadenylation site follows different adenovirus promoters but not with the adenovirus L3 polyadenylation site. The deletion of the two AATAAA copies or of the GT-rich region in the SV40 early polyadenylation site abolishes polyadenylation and also transcription termination.

EXPERIMENTAL PROCEDURES

Construction of Plasmid DNAs

The construction of plasmid DNA containing the adenovirus EIII or EIV promoters upstream of the SV40e polyadenylation site was as follows. Plasmids pEIV(−227/−46) [20] and pEIII [21] that contain the bacterial chloramphenicol acetyl transferase gene (CAT gene) and the SV40e polyadenylation site were digested with endonucleases Hind III or Sst I and Hpa I, respectively, resulting in the removal of only the CAT sequences. The SV40

early polyadenylation recognition elements were retained. Plasmid DNA containing the adenovirus major late-TATA motif upstream of the EIV transcriptional start site and the SV40e polyadenylation site, pML-SVe, was constructed by inserting a synthetic double stranded oligonucleotide containing the Ad-MLP-TATA motif (5'-GCTATAAAAGGG-3' and its complement) to the Bam HI site of plasmid DNA pEIV(−227/−11) [20], which was previously digested with endonucleases Hind III and Hpa I to remove the CAT sequences.

To produce a plasmid DNA containing a deletion of the polyadenylation recognition elements 5'-AATAAA-3' (plasmid-AAUAAA), plasmid pEIV(−227/−46), which contains the CAT coding sequences and the SV40 early polyadenylation site, was digested with the endonucleases HindIII and BsmI. This resulted in the removal of the CAT gene and the two copies of the AATAAA polyadenylation recognition element; however, the cleavage site and the GT rich element of the SV40 early polyadenylation site were kept intact. To generate a construct containing a deletion of the GT-rich region located downstream from the cleavage site (plasmid-GT), construct pEIV-SVe was digested with endonucleases Bsm I and Xba I. The ends were filled in with the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. To construct the mutant pEIV(-polyA), which lacks the entire SV40 early polyadenylation site, plasmid pE4(−227/−46) was digested with Hind III and Xba I. The ends were filled in with the Klenow fragment of DNA polymerase I and ligated with T4 DNA ligase. This resulted in the removal of both the CAT coding sequences and the SV40e polyadenylation site.

Plasmid DNA containing the adenovirus L3 polyadenylation site downstream of the Ad-ML promoter (pML-L3) was constructed as follows. Plasmid DNA pML-SVe was digested with endonucleases Cla I and Hind III. The resulting DNA fragment, which contains the same EIV promoter region as plasmid pEIV (−227/−46), was inserted into plasmid DNA pG3L3-A [22] that was digested with endonucleases Nae I and Hind III.

Plasmid DNA containing the SP6 promoter upstream of the SV40 polyadenylation site (pSP18-SVe) was made as follows. Plasmid pEIV(−227/−46) was digested with endonucleases Hpa I and Xba I to release the complete SV40 early polyadenylation sequence; this sequence was then inserted into Hinc II and Xba I sites of pSP18 (Bethesda Research Laboratories).

Primer Extension Analysis

The oligonucleotides used in the primer extension reaction to analyze the levels of transcription before the polyadenylation cleavage site in the SV40 early polyadenylation region were: (5'-GCAGTAAAAAAGAAAACCTA-3') [BI-EIV] which hybridizes between nucleotides +48 and +68 in the pML-SVe; pEIV-SVe and pEIV-SVe deletion mutants; and (5'-TAAGC-TGCAATAAACAAGTT-3') [BI-SV], which hybridizes between nucleotides +74 and +94 in the pML-SVe, pEIV-SVe and its mutant (-GT). The oligonucleotide used to quantitate the levels of polyadenylation obtained with the SV40 early plasmids was (5'-TTTTTTTTTTGCAGT-3') [polyA] which hybridizes with the last five nucleotides before the cleavage site and the first ten adenosine residues at the poly(A) tail. To measure the levels of transcription after the cleavage site in the SV40 early polyadenylation region the synthetic oligonucleotides used were: (5'-TGGACAAACCACAACTAGAA-3') [AI-SV], which hybridizes between nucleotides +156 and +176 in the pEIV-SVe and pML-SVe; (5'-TAAGATACATTTGATGAGTT-3') [AII-SV], which hybridizes between nucleotides +177 and +197 in the pEIV-SVe and pML-SVe; (5'-CATCACCGAAA-CGCGCGAGG-3') [AIII-VEC], which hybridizes between nucleotides +219 and +239 in the pEIV-SVe and pML-SVe and (5'-GTATACACTCCGCTATCGCT-3') [AIV-VEC], which hybridizes between nucleotides +380 and +400 in the pEIV-SVe and pML-SVe.

The oligonucleotide used to measure transcription before the L3 cleavage site (5'-CGAACGTACGGACGTCCAGC-3') anneals between nucleotides +73 and +93 in the pML-L3. For the quantification of the levels of polyadenylation in the L3 transcript, the oligonucleotide used was (5'-TTTTTTTTTTA-CAAA-3'). Finally, the oligonucleotide used to quantitate the amount of transcription after the cleavage site in the L3 transcript was (5'-GAATTCGAGCTCGGTACCCG-3'), which hybridizes between nucleotides +231 and +251 in the transcripts analyzed.

Transcription reactions *in vitro* were performed as previously described [23] with the exception that 30% (v/v) HeLa cell nuclear extract was used and 2 mM creatine phosphate and 2 mM magnesium were included in the assays for coupling of polyadenylation to transcription. The amount of the different plasmid DNAs per assay was 1.5 μg. After 1 hour incubation at 30°C, reactions were terminated by the addition of an equal volume of stop solution: 10 μg/ml sonicated salmon sperm DNA; 10 mM EDTA; 100 mM Na-acetate and 0.2% SDS. After phenol/chloroform extraction and precipitation of the transcript, it was resuspended in 25 μl of 2×hybridization buffer [250 mM KCl; 1 mM EDTA and 10 mM Tris-HCl, pH 7.5, containing c.a. 100,000 cpm/ng of 32-P-labeled oligonucleotide], incubated for 10 min. at 65°C, and then cooled to 42°C. After the addition of 25 μl of 2×reverse transcriptase buffer [20 mM $MgCl_2$; 4 mM dithiothreitol; 0.5 mM EDTA; 40 mM Tris-HCl, pH 8.3 and 250 μM of all four dNTPs containing 200 units avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories)], the reaction mixtures were incubated at 42°C for 1 h.

RESULTS

Transcription-polyadenylation in HeLa cell nuclear extracts

In order to analyze the effect of poly A addition recognition sequences during elongation by RNA polymerase II, constructs containing different polyadenylation sites downstream of different adenovirus promoters were produced. These constructs were transcribed in HeLa cell extracts and the amount of transcription 5' and 3' of the polyadenylation cleavage site, as well as the levels of polyadenylation were determined using oligonucleotides complementary to different parts of the body of the nascent RNA, to prime reverse transcriptase (for details see Fig 1).

It has been previously demonstrated that the conserved hexanucleotide AAUAAA [24−27] and the GU-rich region [28−32] present in the SV40e polyadenylation site are necessary for poly A addition *in vivo* and *in vitro*. In order to analyze whether the polyadenylation activity observed under transcription conditions was dependent on such DNA elements, these sites were deleted. Polyadenylation was measured using an oligonucleotide composed of five residues complementary to those preceding the SV40 early cleavage site followed by ten thymidine residues. This oligonucleotide permitted measuring polyadenylation specifically (Fig. 2A, lanes 1 and 2); an oligonucleotide that replaced the

thymidine residues by ten guanidylic residues failed to score for polyadenylated molecules (data not shown).

The removal of the two copies of the hexanucleotide AATAAA present in the SV40e polyadenylation site or of the GT-rich region resulted in constructs capable of transcription but incapable of polyadenylation (Fig. 2A, compare lanes 1 and 2 with 3 and 4 and 5 and 6, respectively). The size of the polyadenylated molecules (lanes 1 and 2) is in agreement with the size expected for an RNA molecule that was both accurately initiated at the EIV promoter and polyadenylated at the SV40e polyadenylation site (see Fig. 1 for details). In agreement with the results of Moore and Sharp [33], the adenovirus L3 polyadenylation site was functional in the assay conditions (Fig. 2B, lanes 1–3). Also, poly A addition was dependent on an active polyadenylation site but independent of the promoter used to drive transcription, as significant levels of polyadenylation at the SV40e site were observed when transcription was directed by either the adenovirus EIV (Fig. 2A, lanes 1 and 2), major late (Fig. 2C, lanes 1 and 2) or EIII (Fig 2C, lanes 3 and 4) promoters. However, the efficiency of polyadenylation appears to vary with the promoter used; higher levels of polyadenylated RNA were observed when the Ad-EIII promoter directed transcription through the SV40e

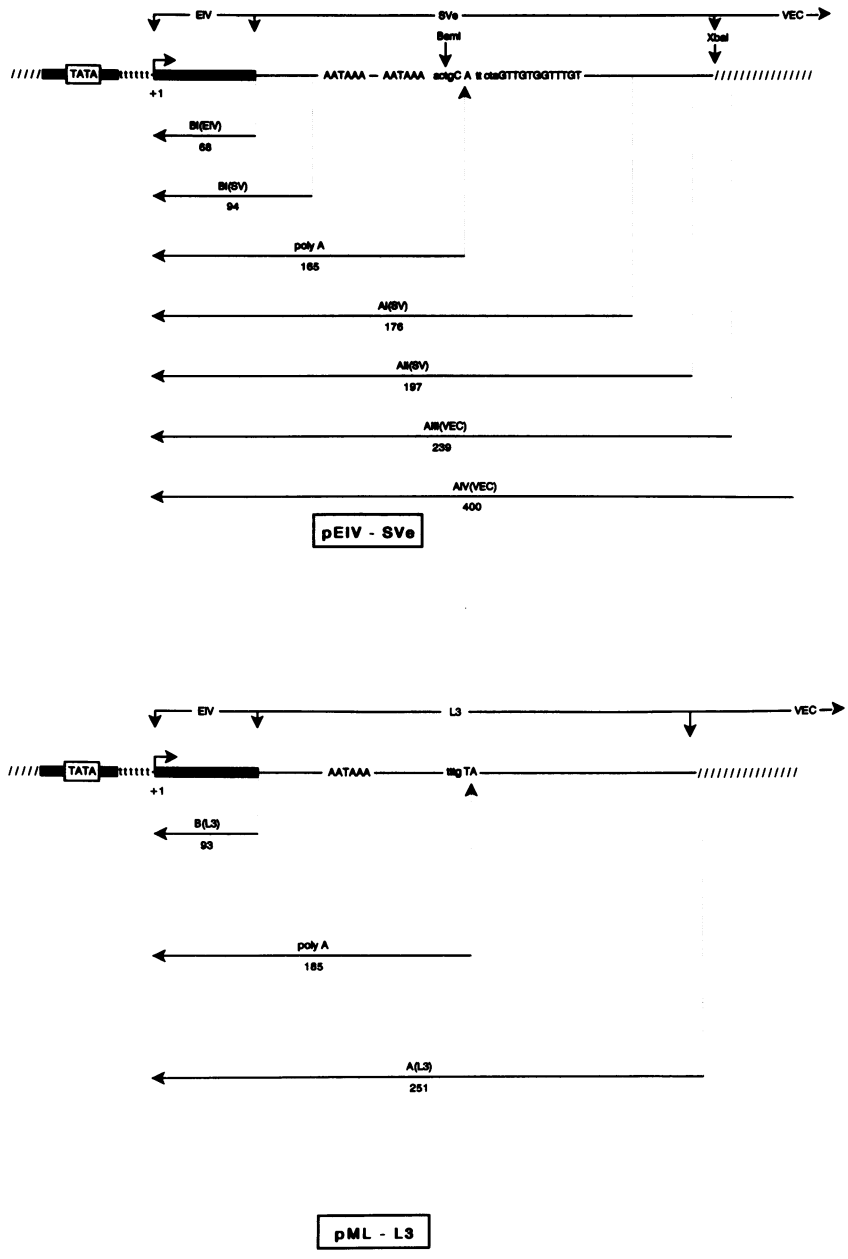

Figure 1. Schematic representation of pEIV-SVe and pML-L3 constructs indicating the size of the transcripts generated with the different primers used in the polyadenylation coupled to transcription assays. The construction of these plasmids is described under 'Experimental Procedures'. The letters on the arrows indicate the name of the primer used in the primer-extension analysis and the numbers below the arrows indicate the predicted size for a primer extended molecule that was initiated at the +1 site utilized *in vivo*. DNA elements present in the SV40 early and L3 polyadenylation regions are indicated as well as sites for relevant restriction endonucleases. The arrow between C and A represents the cleavage site for the SV40 early and the arrow between T and A represents the cleavage site for the L3 polyadenylation sequence.

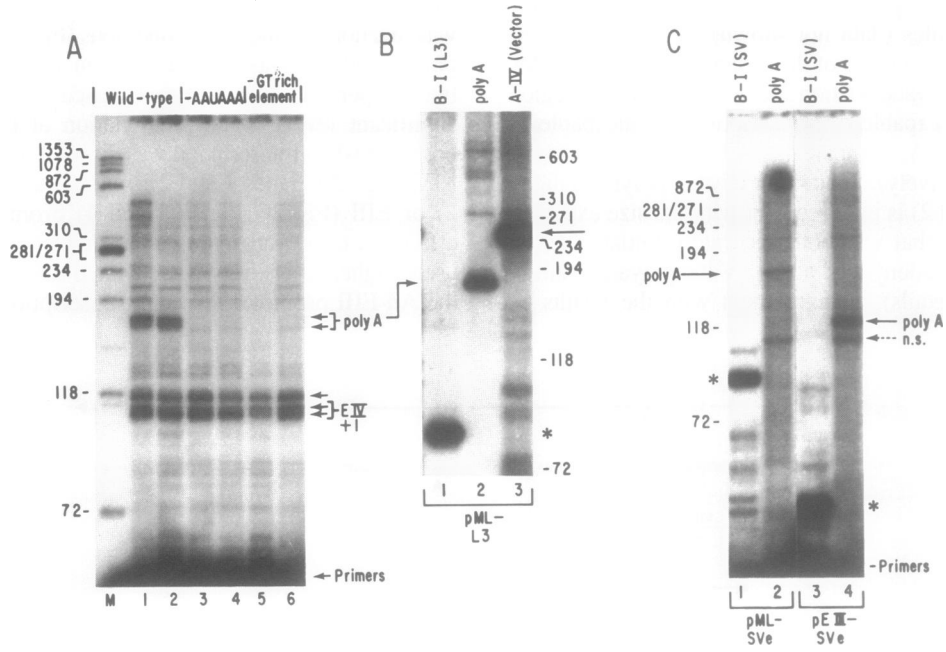

Figure 2. In vitro polyadenylation of different adenovirus promoters with different polyadenylation sites. (A). Reaction conditions were as described in Experimental Procedures including pEIV-SVe circular plasmid DNA (lanes 1 and 2), mutant (-AAUAAA) circular plasmid DNA (lanes 3 and 4), and mutant (-GT) circular plasmid DNA (lanes 5 and 6). All the reactions include 1.5 μg of pEIV(−227/−46) circular plasmid DNA as an internal control. After 1hr. at 30°C; oligonucleotide poly(A): [5′-TTTTTTTTTTGCAGT-3′] was used to analyze polyadenylation of the synthesized transcripts. Oligonucleotide CAT: [5′-CTAAGGAAGCTAAAATGGAG-3′], which hybridizes with the pEIV(−227/−46) transcript but not with either the pEIV-SVe transcript or the mutant transcripts, was used to analyze the transcription of the internal control. Lanes 1, 3 and 5 correspond to an incubation using a nuclear extract preparation different from that in lanes 2, 4 and 6. (B) pML-L3 circular plasmid DNA was transcribed *in vitro* . After 1 hr. at 30°C, oligonucleotide [5′-CGAACGTACGGACGTCCAGC-3′] (lane1), oligonucleotide [5′-TTTTTTTTTTA-CAAA-3′] (lane 2) and oligonucleotide [5′-GAATTCGAGCTCGGTACCCG-3′] (lane 3) were used to analyze the levels of transcription before the cleavage site (lane 1); polyadenylation (lane 2) and transcription after the cleavage site (lane 3), by primer extension. (C) pML-SVe (lanes 1 and 2) and pEIII-SVe (lanes 3 and 4) circular plasmid DNAs were transcribed *in vitro* . After 1 hr. at 30°C, oligonucleotide (BI-SV): [5′-TAAGCTGAATAAACAAGTT-3′] (lanes 1 and 3) and oligonucleotide (polyA): [5′-TTTTTTTTTTGCAGT-3′] were used to analyze the levels of transcription (*) and polyadenylation, respectively, by primer extension. The extended molecules were separated by electrophoresis on an 8% polyacrylamide-urea regular gel.

polyadenylation site followed by the Ad-EIV promoter and the Ad-MLP. The significance of these differences, if any, is currently unknown.

The SV40 early polyadenylation site but not the adenovirus L3 polyadenylation site affects elongation by RNA polymerase II

In order to analyze the effect of a polyadenylation site during elongation by RNA polymerase II after accurately initiating transcription, the level of RNA 5′ and 3′ of a polyadenylation site were measured using reverse transcriptase and oligonucleotides complementary to different regions of the RNA transcript (for details see Fig 1).

The level of RNA observed from a construct DNA containing the adenovirus major late promoter and directing transcription through the L3 polyadenylation site was approximately the same when measured 5′ or 3′ of the Ad-L3 polyadenylation site (Fig. 2B, compare lane 1 with 3). These results are in agreement with those of Moore and Sharp [33], which observed that the L3 poly(A) site had no effect on elongation by RNA polymerase II. Interestingly, a different situation was observed when the Ad-EIV promoter directed transcription through the SV40 early poly (A) addition site. Under these conditions the levels of RNA detected 3′ from the poly (A) site were lower than those detected 5′ from the site. The level of RNA appears to decrease when the primer used for reverse transcriptase hybridized to sequences located downstream from the polyadenylation-cleavage site (Fig. 3 compare lane 1 with lanes 2, 3, 4 and 5). The observed effect on elongation by RNA polymerase II was not artifactual or due to differences in the specific activity of the primer used for reverse transcriptase. Primer A-III (vector), which gave lower levels of activity when used to analyze transcription from a plasmid containing the SV40e polyadenylation site (Fig. 3A, lane 4), yielded, when the sequences required for polyadenylation were deleted, levels of transcription similar to those observed with a primer that hybridized 5′ of the polyadenylation site (Fig. 3A, compare lane 6 with 1, respectively). Also, a transcript generated using the SP6 polymerase and containing the complete SV40 early polyadenylation sequence gave similar levels of activity when analyzed using oligonucleotides BI(SV), AI(SV) and AII(SV) (see Fig. 4), eliminating the possibility that the differences observed in Fig. 3 were due to differences in the specific activity of the primers used in the analysis by primer extension. Thus, the observed effect on elongation by RNA polymerase II was dependent on the SV40e polyadenylation site. The observed effect was independent of the promoter used, as a decrease in transcription 3′ of the SV40 polyadenylation site was also observed when the Ad-MLP directed transcription through the SV40e polyadenylation sequences (Fig. 3B).

In the previous section, and in agreement with published studies, polyadenylation was shown to be dependent on the AATAAA and GT-rich DNA motifs. Next we analyzed whether the observed effect of the SV40e polyadenylation sequences on elongation by RNA polymerase II was dependent on the

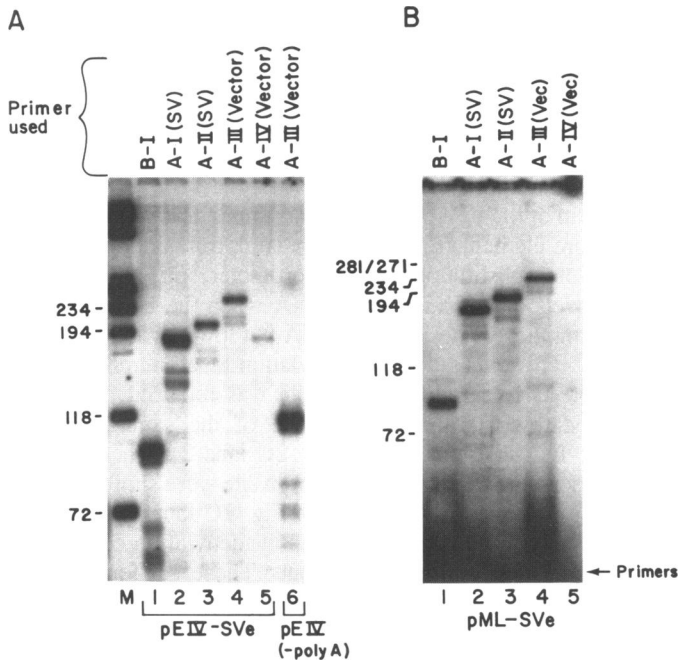

Figure 3. Transcription termination is a gradual process after the polymerase passes through the cleavage site. (A) pEIV-SVe (lanes 1–5) or pEIV-(polyA) (lane 6) circular plasmid DNAs were transcribed as described in Experimental Procedures . After 1 hr. at 30°C, oligonucleotide (BI-SV): [5′-TAAGCTGCA-ATAAACAAGTT-3′] (lane 1), oligonucleotide (AI-SV): [5′-TGGACAAACC-ACAACTAGAA-3′] (lane 2), oligonucleotide (AII-SEV): [5′-TAAGATACA-TTTGATGAGTT-3′] (lane 3), oligonucleotide (AIII-VEC): [5′-CATCACCG-AAACGCGCGAGG-3′] (lane 4 and 6), and oligonucleotide (AIV-VEC) 5′-GTATACACTCCGCTATCGCT-3′ (lane 5) were used to analyze the levels of transcription before and after the cleavage site, by primer extension. (B) pML-SVe was analyzed using the same conditions as those used in panel A for pEIV-SVe.

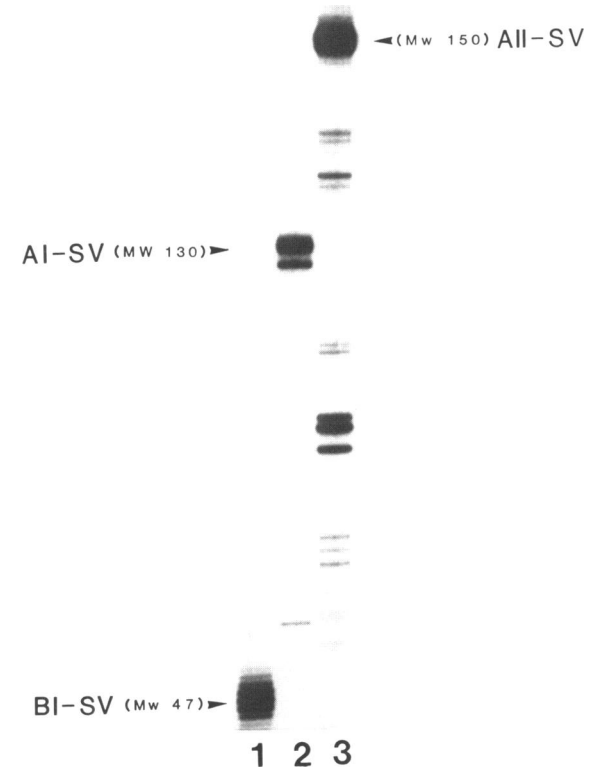

Figure 4. The levels of transcription 5′ and 3′ to the cleavage site are the same in a transcript synthesized using the SP6 system. pSP18-SVe transcript was synthesized as described previously [36]. Oligonucleotide (BI-SV): [5′-TAAGC-TGCAATAAACAAGTT-3′] (lane 1), oligonucleotide (AI-SV): [5′-TGGACA-AACCACAACTAGAA-3′] (lane 2) and oligonucleotide (AII-SV): [5′-TAAGATAACTTTGATGAGTT-3′] (lane 3) were used to analyze the level of transcription before and after the SV40e cleavage site, by primer extension. The extended molecules were separated by electrophoresis on an 8% polyacrylamide-urea sequencing gel.

AATAAA motif. DNA constructs containing the adenovirus EIV promoter and directing transcription through the wild-type SV40e polyadenylation site or through a polyadenylation site containing a deletion of the AATAAA were analyzed as described above. The levels of transcription from the Ad-EIV promoter were approximately the same in the two constructs analyzed, as determined by using an oligonucleotide (BI-EIV) that hybridized 70 nucleotides downstream of the transcriptional start site in the EIV coding sequences (Fig. 5, compare lanes 4 with 8). The approximately 70 nucleotide products position the start site of transcription at or near the run of T residues. This run of T residues is used *in vivo* as the start site of transcription. Consistent with the results presented above (Fig. 3), the level of transcription decreased drastically when the polymerase passed through the polyadenylation site (Fig. 5, compare band +1 in lane 4 with bands a′, b′, and c′ in lanes 1–3). However, when the AATAAA motifs were deleted, the level of transcription downstream of the polyadenylation cleavage site remained proportional to the level of transcription prior the polyadenylation site (Fig 5, compare band +1 in lane 8 with bands a, b, and c in lanes 4–7). Similar results were observed when the GT-rich element was deleted (data not shown). The nature of the small primer extended molecules in lanes 3 and 7 is currently unknown. It is possible that they represent pausing sites for the reverse transcriptase, so that the levels of transcription observed when the primer AIV-VEC is used to measure the transcription 3′ to the cleavage site are underestimated.

These results indicate that the AATAAA as well as the GT-rich motifs were both necessary to affect elongation of RNA polymerase II and are in agreement with *in vivo* studies demonstrating that both the AATAAA and the GT-rich elements were necessary for transcription termination [19]. The results of the *in vitro* system indicate that the polymerase is affected immediately after the polyadenylation site. However, studies using the β-major globin polyadenylation site indicated that the elongating polymerase could continue elongation for hundreds of nucleotides 3′ of the polyadenylation site prior to complete termination [34]. These differences remain to be further studied.

DISCUSSION

The first experiments to study *in vitro* polyadenylation of mRNA precursors in a HeLa whole-cell lysate were carried out by Manley [35]. The precursors were synthesized by an *in vitro* transcription reaction, using a linear DNA template in whole-cell extract. After purification, these precursors were incubated in another lysate to become polyadenylated RNA molecules. Under optimal conditions, 70% of the pre-mRNA was polyadenylated.

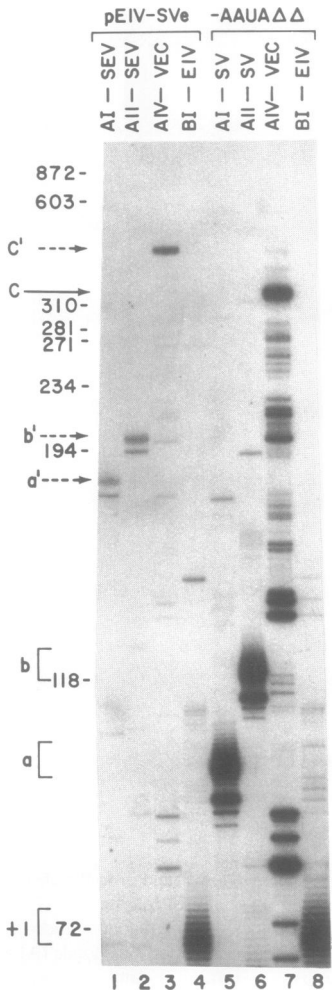

Figure 5. Transcription termination in pEIV-SVe transcript and mutants thereof. Transcription reactions were performed as described in Experimental Procedures. pEIV-SVe circular plasmid DNA (lanes 1,2,3 and 4) and (-AAUAAA) deletion mutant circular plasmid DNA (lanes 5,6,7 and 8) were transcribed for 1 hr. at 30°C. Oligonucleotide (BI-EIV): [5'-GCAGTAAAAAAGAAAACCTA-3'] (lanes 4 and 8), oligonucleotide (AI-SV): [5'-TGGACAAACCACAACTAGAA-3'] (lanes 1 and 5), oligonucleotide (AII-SV): [5'-TAAGATACATTTGATG-AGTT-3'] (lanes 2 and 6), and oligonucleotide (AIV-VEC): [5'GTATACACT-CCGCTATCGCT-3'] (lanes 3 and 7), were used to analyze the level of transcription before and after the cleavage site, by primer extension. The extended molecules were separated by electrophoresis on a 5% polyacrylamide-urea sequencing gel.

Since then, most *in vitro* studies in polyadenylation have utilized the SP6 system [36] to generate transcripts. These transcripts, upon isolation, are used as substratres in *in vitro* polyadenylation systems with nuclear extracts.

The development of these systems has permitted the identification of factors [37−42] and cis-acting sequence elements [26−32] which are involved in poly(A) addition. To date, with the exception of the system described by Moore and Sharp [33], an *in vitro* system to study polyadenylation coupled to transcription directed by class II promoters has not been developed.

A coupled polyadenylation-transcription *in vitro* system has been described here which allows the synthesis of transcripts containing different polyadenylation sites. The transcription of RNA driven by different adenovirus promoters is analyzed by reverse transcription employing different primers which hybridize before or after the polyadenylation cleavage site or with the poly(A) tail in the polyadenylated molecules.

The results presented in Fig. 2 show that the two copies of the hexanucleotide AATAAA and the GT-rich region present in the SV 40 early polyadenylation site are required for *in vitro* polyadenylation as was shown previously [15 for review]. The substitution of the EIV promoter by a promoter that contains the TATA core element from the major late promoter, pML-SVe, or complete substitution by EIII promoter, pEIII-SVe, gave similar levels of polyadenylated RNA when the EIII promoter was driving the synthesis of the RNA but lower with pML-SVe (Fig. 2C). However, the polyadenylation level increased when the SV 40 early polyadenylation site was replaced by the L3 polyadenylation site in the presence of the same promoter (compare Fig. 2B lane 2 with Fig. 2C lane 2). The levels of transcription before the polyadenylation site were similar in both cases (compare Fig. 2B lane 1 with Fig. 2C lane 1) ruling out the possibility that lower levels of polyadenylation correspond to lower levels of transcription. These differences in polyadenylation between the SV 40 early region and the L3 have been previously observed *in vitro* by others [28,38 and 43]. Accurate polyadenylated molecules in constructs containing polyadenylation signals different from the SV 40 early site have also been observed here. EIII promoter with either the L3 or E2 polyadenylation site produces accurately polyadenylated RNAs . The same results are obtained with the EIV promoter and the E2 polyadenylation site (data not shown).

Two possible mechanisms have been proposed for transcription termination [7]. In one of these, the poly(A) region plays a crucial role in the termination process; in the other, different or similar DNA sequences act as termination signals for the polymerase [8−14]. This last mechanism could apply for at least some histone genes which, although transcribed by the RNA polymerase II, do not possess poly(A) sequences. Three different and separate sequences are required in the sea urchin H2A gene for their transcription termination [10]. One of these sequences is a part of the coding region, the other two are in the 3′ flanking region of the gene. One of the sequences in the 3′ region contains several oligo(T)-rich regions. These regions are homologous to that at the 3′ end of the human gastrin gene which is also involved in transcription termination [8]. This oligo(T)-rich sequence resembles some AT-rich sequences in yeast, as the TTTTATA sequence which is involved in Saccharomyces cerevisiae RNA polymerase termination [44−45]. The final 3′ end of histone mRNA is formed by a 3′ processing mechanism mediated by small nuclear RNPs [6−7].

Two different models have been proposed for transcription termination in class II genes with poly(A) regions. One model suggests that the 3′ cleavage of the nascent pre-mRNA triggers the termination. When the RNA polymerase II in the transcription complex passes through the poly(A) region, cleavage of the RNA molecule occurs, generating an uncapped 5′ transcript. The polymerase continues to transcribe, but the new 5′ unprotected end is rapidly degraded, destabilizing the transcription complex and permiting the RNA polymerase to release the complex [6,7].

In the other model, the complex has an antiterminator factor which is released from the complex when this reaches the polyadenylation site resulting in termination of the transcription [17]. These results support the second model rather than the first because there is no degradation 5′ to 3′ of the uncapped 5′ transcript generated after the cleavage and polyadenylation

reaction (compare lane 2 with lanes 3 to 5 in Fig. 3A and compare the same lanes in Fig. 3B) . It appears that a functional polyadenylation site is required for polyadenylation as well as transcription termination as previously described [16, 17 and 19]. These results are in agreement with those of Connelly and Manley [19] who found that the deletion of either both AATAAA sequence elements or the GT-rich region in the SV40 early polyadenylation site produces increasing amounts of read-through RNA in comparison with the wild type plasmid. This read-through transcription represents transcription initiated at the +1 which passes through the polyadenylation region and continues over the circular plasmid.

From these results, transcriptional termination seems to be a gradual process. After the release of a possible antiterminator factor from the transcriptional machinery, transcription continues at a lower efficiency until the polymerase recognizes a termination sequence or until so few molecules of polymerase remain associated with the template that termination is considered to be terminated. This may explain why some groups [34,46] suggest that transcription termination occurs at many different sites on the DNA. In the L3 polyadenylation sequence, perhaps other factors, such as the small nuclear ribonucleoproteins involved in the cleavage of histone mRNAs and in the cleavage of some mRNAs [47], or sequences present in the gene itself, such as the CCAAT box sequence in the adenovirus major late promoter which functions as a termination signal [13,14], are required, together with L3 polyadenylation sequence, to permit transcription termination. The information required to initiate the termination process, may also be contained in the secondary structure of the RNA and could be the reason of the difference observed between the L3 and the SV40 early polyadenylation sites.

Furthermore, the existence of different polyadenylation sites within the same transcription unit, while perhaps implying a regulatory role for the polyadenylation event in the gene expression and resulting in the synthesis of different forms of the same protein [48], further substantiates a mechanism which permits the transcription termination when the polymerase passes through certain polyadenylation sites but not when it passes through others.

ACKNOWLEDGMENTS

I wish to thank E.Maldonado, M.J.Agulló, P.Morales and Dr. J.Saus for helping me in different phases of this work; L.Weiss and Dr. F.Thompson for critically reading the manuscript and Dr. J.L.Manley for providing me with plasmid pG3L3-A. I also thank Dr. D.Reinberg for support while working on this project in his laboratory. This work was partially supported by grants F.I.S. 89/0406 and C.I.C.Y.T. PB87/0951. V.J.Miralles was a recipient of a fellowship from NATO-Spain and now is a postdoctoral fellow of the Ministerio de Educación y Ciencia of Spain.

REFERENCES

1. Furuichi, Y., La Fiandra, A. and Shatkin, A. (1977) Nature, **226**, 235–239.
2. Lockard, R. and Lane, C. (1978) Nucleic Acids Res., **5**, 3237–3247.
3. Konarska, M.M., Padget, R.A. and Sharp, P.A. (1984) Cell, **38**, 731–736.
4. Edery, I. and Sonenberg, N. (1985) Proc. Natl. Acad. Sci. USA, **82**, 7590–7594.
5. Darnell, J.E. Jr. (1982) Nature, **297**, 365–371.
6. Birnstiel, M.L., Busslinger, M. and Strub, K. (1985) Cell, **41**, 349–359.
7. Proudfoot, N.J. (1989) Trends Biochem. Sci., **14**, 105–110.
8. Sato, K., Ito, R., Baek, K-H. and Agarwal, K. (1986) Mol. Cell. Biol., **6**, 1032–1043.
9. Baek, K-H., Sato, K., Ito, R. and Agarwal, K. (1986) Proc. Natl. Acad. Sci. USA., **83**, 7623–7627.
10. Johnson, M.R., Norman, C., Reeve, M.A., Scully, J. and Proudfoot, N.J. (1986) Mol. Cell. Biol., **6**, 4008–4018.
11. Law, R., Kuwabara, M.D., Briskin, M., Fasel, N., Hermanson, G., Sigman, D.S. and Wall, R. (1987) Proc. Natl. Acad. Sci. USA, **84**, 9160–9164.
12. Pribyl, T.M. and Martinson, H.G. (1988) Mol. Cell. Biol., **8**, 5369–5377.
13. Connelly, S. and Manley, J.L. (1989) Cell, **57**, 561–571.
14. Connelly, S. and Manley, J.L. (1989) Mol. Cell. Biol., **9**, 5254–5259.
15. Manley, J.L. (1988) Biochim. Biophys. Acta, **950**, 1–12.
16. Whitelaw, E. and Proudfoot, N.J. (1986) EMBO J., **5**, 2915–2922.
17. Logan, J., Falck-Pedersen, E., Darnell, J.E. and Shenk, T. (1987) Proc. Natl. Acad. Sci. USA, **84**, 8306–8310.
18. Lanoix, J. and Acheson, N.H. (1988) EMBO J., **7**, 2515–2522.
19. Connelly, S. and Manley, J.L. (1988) Genes Dev., **2**, 440–452.
20. Leza, A. and Hearing, P. (1988) J. Virol., **62**, 3003–3013.
21. Weeks, D.L. and Jones, N.C. (1985) Nucleic Acids Res., **13**, 5389–5402.
22. Takagaki, Y., Ryner, L.C. and Manley, J.L. (1988) Cell, **52**, 731–742.
23. Miralles, V.J., Cortes, P., Stone, N. and Reinberg, D. (1989) J. Biol. Chem., **264**, 10763–10772.
24. Manley, J.L., Yu, H. and Ryner, L. (1985) Mol. Cell. Biol., **5**, 1347–1368.
25. Moore, C.L., Skolnik-David, M. and Sharp, P.A. (1986) EMBO J., **5**, 1929–1938.
26. Zarkower, D., Stephenson, P., Sheets, M. and Wickens, M. (1986) Mol. Cell. Biol., **6**, 2317–2323.
27. Conway, L. and Wickens, M. (1987) EMBO J., **6**, 4177–4184.
28. Hart, R.P., McDevitt, M.A. and Nevins,J.R. (1985) Cell, **43**, 677–683.
29. Sperry, A.O. and Berget, S.M. (1986) Mol. Cell. Biol., **6**, 4734–4741.
30. Green, T.L. and Hart, R.P. (1988) Mol. Cell. Biol., **8**, 1839–1841.
31. Zarkower, D. and Wickens, M. (1988) J. Biol. Chem., **263**, 5780–5788.
32. Ryner, L.C., Takagaki, Y. and Manley, J.L. (1989) Mol. Cell. Biol., **9**, 1759–1771.
33. Moore, C.L. and Sharp, P.A. (1984) Cell, **36**, 581–591.
34. Citron, B., Falck-Pedersen, E., Salditt-Georgieff, M. and Darnell Jr., J.E. (1984) Nucl. Acids Res., **12**, 8723–8731.
35. Manley, J.L. (1983) Cell, **33**, 595–605.
36. Melton, D., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res., **12**, 7035–7056.
37. Christofori, G. and Keller, W. (1988) Cell, **54**, 875–889.
38. Christofori, G. and Keller, W. (1989) Mol. Cell. Biol., **9**, 193–203.
39. Gilmartin, G., McDevitt, M.A. and Nevins, J.R. (1988) Genes Dev., **2**, 578–587.
40. McDevitt, M.A., Gilmartin, G.A. Reeves, W.H. and Nevins, J.R. (1988) Genes Dev., **2**, 588–597.
41. Sheets, M.D. and Wickens, M. (1989) Genes Dev., **3**, 1401–1412.
42. Gilmartin, G. M. and Nevins, J.R. (1989) Genes Dev., **3**, 2180–2189.
43. Hashimoto, C. and Steitz, J.A. (1986) Cell, **45**, 581–591.
44. Henikoff, S., Kelly, J.D. and Cohen, E.H. (1983) Cell, **33**, 607–614.
45. Henikoff, S. and Cohen, E.H. (1984) Mol. Cell. Biol., **4**, 1515–1520.
46. Hagenbuchle, O., Wellauer, P.K., Cribbs, D.L. and Schibler, U. (1984) Cell, **38**, 737–744.
47. Mowry, K.L. and Steitz, J.A. (1988) Trends Biochem. Sci., **13**, 447–451.
48. Denner, D. and Leder, P. (1985) Proc. Natl. Acad Sci. USA., **82**, 8658–8662.