MOLECULAR AND CELLULAR BIOLOGY, June 1996, p. 2579–2584 Vol. 16, No. 6
0270-7306/96/$04.00+0

The Cap and the 3′ Splice Site Similarly Affect Polyadenylation Efficiency

CHARLES COOKE AND JAMES C. ALWINE*

Graduate Group of Molecular Biology and Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6142

Received 11 December 1995/Returned for modification 31 January 1996/Accepted 28 February 1996

The 5′ cap of a mammalian pre-mRNA has been shown to interact with splicing components at the adjacent 5′ splice site for processing of the first exon and the removal of the first intron (E. Izaurralde, J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz, and I. W. Mattaj, Cell 78:657–668, 1994). Likewise, it has been shown that processing of the last exon and removal of the last intron involve interaction between splicing components at the 3′ splice site and the polyadenylation complex at the polyadenylation signal (M. Niwa, S. D. Rose, and S. M. Berget, Genes Dev. 4:1552–1559, 1990; M. Niwa and S. M. Berget, Genes Dev. 5:2086–2095, 1991). These findings suggest that the cap provides a function in first exon processing which is similar to the function of the 3′ splice site in last exon processing. To determine whether caps and 3′ splice sites function similarly, we compared the effects of the cap and the 3′ splice site on the in vitro utilization of the simian virus 40 late polyadenylation signal. We show that the presence of a m^7GpppG cap, but not a cap analog, can positively affect the efficiency of polyadenylation of a polyadenylation-only substrate. Cap analogs do not stimulate polyadenylation because they fail to bind titratable cap-binding factors. The failure of cap analogs to stimulate polyadenylation can be overcome if a 3′ splice site is present upstream of the polyadenylation signal. These data indicate that factors interacting with the cap or the 3′ splice site function similarly to affect polyadenylation efficiency and complete exon processing. We also find that a 5′ splice site directly upstream of the polyadenylation signal, along with a m^7GpppG cap, is inhibitory to polyadenylation. This finding suggests that the interaction between the cap-binding complexes and splicing components at the 5′ splice site may form a complex which is inhibitory to further processing if splicing of an adjacent intron is not achieved.

The majority of pre-mRNAs in mammalian cells are processed in the nucleus by 7-methylguanosine (m^7GpppG) cap formation on the 5′ end and splicing and polyadenylation on the 3′ end. The biochemistry of each of these individual processing reactions has been extensively studied (9, 10, 13, 16, 27, 29). These studies suggest that in the cell, the processing of an mRNA is highly coordinated and that regulatory interactions must occur between the specialized factors which mediate each individual processing reaction. Such coordination has been suggested in the exon definition model proposed by Berget (2).

According to the exon definition model (Fig. 1), mammalian interior exons, because of their small size (average of 137 nucleotides), are defined when the splicing factors locate a pair of closely spaced splice sites in exonic polarity (3′ splice site-exon sequences-5′ splice site). This localization of splice sites allows exon definition by the binding of U1 and U2 small nuclear ribonucleoproteins (snRNPs) and associated factors (2). However, the two terminal exons require a different mechanism given that the first exon has a cap structure instead of a 3′ splice site and the last exon has a polyadenylation signal instead of a 5′ splice site.

The mechanism for the processing of the first exon has been suggested by the work of Izaurralde et al. (9), which showed that an m^7GpppG cap and a nuclear cap-binding complex (CBC; an 80-kDa and a 20-kDa cap-binding protein) were essential for efficient in vitro removal of the adjacent intron, using a simple one-intron substrate. These data suggest that an early stage of spliceosome assembly, possibly the formation of the commitment complex, is blocked by either the use of a cap analog (to which CBC does not bind) or the depletion of the CBC from splicing extracts. These data suggest that the CBC, in association with the m^7GpppG cap, may interact with components of the commitment complex (Fig. 1), providing a means to define the exon or to activate the progression to full spliceosome assembly. This effect of the cap and the CBC appears to affect only the removal of the first intron. Previous in vitro experiments using a two-intron system indicated that changing the m^7GpppG cap to an adenosine inhibited removal of the first intron but had little or no effect on the removal of the second (22).

Several lines of experimentation have suggested that processing of the last exon involves interaction with splicing components at the 3′ splice site and the polyadenylation complex (Fig. 1). Such interactions have been suggested by experiments using a coupled in vitro splicing and polyadenylation system (19, 21). These data showed that mutations in the polyadenylation signal which inhibited polyadenylation also caused a decrease in the efficiency of splicing, i.e., removal of the last intron. Likewise, mutations in the 3′ splice site of the last exon, which inhibit splicing, caused inhibition of polyadenylation. Analogous transfection experiments have provided similar results in vivo (4, 17, 18). Several previous reports have suggested a role for a major splicing component, the U1 snRNP, in nuclear polyadenylation (8, 14, 23, 28). In addition, our laboratory has shown that U1 snRNP A protein positively affects the efficiency of polyadenylation of the simian virus 40 (SV40) late polyadenylation signal through interactions with upstream elements (11) as well as direct interactions with components of the cleavage-polyadenylation specificity factor (12).

A significant question is: do mechanisms for 3′- and 5′-terminal exon definition require unique factors, or do similar

* Corresponding author. Mailing address: Cancer Center, 560 Clinical Research Building, 415 Curie Blvd., University of Pennsylvania, Philadelphia, PA 19104-6142. Phone: (215) 898-3256. Fax: (215) 573-3888. Electronic mail address: alwine@mail.med.upenn.edu.

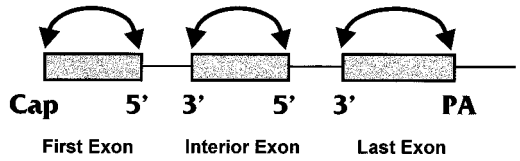

FIG. 1. Definition of exons. The model indicates proposed interactions of factors at the cap, the 5′ and 3′ splice sites, and the polyadenylation signal (PA) which may mediate the processing of first, interior, and last exons of a mammalian precursor RNA.

factors accommodate multiple processing signals, i.e., cap, 3′ and 5′ splice sites, and polyadenylation signal? The data discussed above suggest that for the definition of the first exon, the cap and the bound CBC provide the same function as a 3′ splice site does in interior and last exons; thus, caps and 3′ splice sites may function similarly for exon definition. To test this possibility, we compared the effects of the cap and the 3′ splice site on the in vitro utilization of the SV40 late polyadenylation signal in HeLa cell nuclear extracts. We show that an m^7GpppG cap can positively affect the efficiency of polyadenylation of a polyadenylation-only (PA-only) substrate and that cap analogs do not stimulate because they fail to bind specific cap-binding factors. The failure of cap analogs to stimulate polyadenylation can be overcome if a 3′ splice site is present upstream of the polyadenylation signal. One explanation for these data is that the effects of the cap and the 3′ splice site on polyadenylation may be mediated by similar factors. We also show that a 5′ splice site directly upstream of the polyadenylation signal, along with an m^7GpppG cap, is inhibitory to polyadenylation. This finding suggests that the interaction between the CBCs and splicing components at the 5′ splice site of the first exon may form a complex which is inhibitory to further processing if splicing of an adjacent intron is not achieved.

MATERIALS AND METHODS

Plasmids encoding precursor RNA substrates. Substrate RNAs for in vitro polyadenylation reactions (diagrammed in Fig. 2) were prepared by in vitro transcription from plasmid templates containing the SV40 late polyadenylation signal or from templates containing an adenovirus splicing cassette upstream of the SV40 late polyadenylation signal. Plasmid pGem2-UPAS contains SV40 nucleotides 2533 to 2770 inserted between the *Bam*HI and *Eco*RI sites of pGem2 (Promega Biotec) (26). It produces a substrate representing the wild-type SV40 late polyadenylation signal (WT PA only). Plasmid pGem2-UM123 has been previously described (26); it was derived from pGem2-UPAS and contains linker substitution mutations (*Bgl*II, *Sma*I, and *Bgl*II) in the three repeated upstream efficiency elements of the SV40 late polyadenylation signal.

Plasmid pSP64-MXSVL, which encodes a precursor RNA containing the wild-type SV40 polyadenylation signal fused to a functional adenovirus splicing cassette (WT Sp/PA), has been previously described (19, 21). Several variants of pSP64-MXSVL which produce substrates with mutations in the splicing cassette were used. Plasmid pSP64-MXSVL 3′-ss, which has been previously described (19, 21), produces a substrate which bears a point mutation inactivating the 3′ splice site (-3′ Sp/PA). Plasmid pGem2-5′SpPA produces a substrate which lacks the 5′ exonic sequences (-5′ Sp/PA); it was derived from pSP64-MXSVL by cleavage with *Pst*I, isolation of the resulting 380-nucleotide fragment, and insertion of the fragment into the *Pst*I site of pGem2. Plasmid pGem2-5′-3′Sp/PA produces a substrate lacking the 5′ exonic sequences and containing the point mutation in the 3′ splice sites (-3′,-5′ Sp/PA). It was produced in a manner identical to that used to produce pGem2-5′SpPA except pSP64-MXSVL3′-ss was digested with *Pst*I. Orientation respective to the SP6 or T7 promoter of pGem2 was determined by restriction analysis, and clones which would produce sense RNA from the T7 promoter were chosen for use.

Preparation of precursor RNA substrates. Templates for in vitro transcription were linearized with *Dra*I. Reactions were performed with standard conditions (1), using T7 or SP6 polymerase (Promega Biotec), 50 μCi of [^{32}P]UTP (Amersham), 250 ng of linearized template, and 0.5 mM of cap substrate (m^7GpppG) or cap analog substrate (ApppG [Pharmacia] [9] or GMP-PCP [CalBiochem] [6]). ^{32}P-labeled RNAs were extracted with phenol-chloroform-isoamyl alcohol (50:49:1), ethanol precipitated, and purified by electrophoresis through a 5% polyacrylamide–7 M urea gel. RNAs were eluted from the gel slices in 20 mM Tris (pH 7.5)–400 mM NaCl–0.01% sodium dodecyl sulfate at room temperature overnight. After extractions with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol (49:1), RNAs were ethanol precipitated. Incorporated [^{32}P]UTP was quantitated by liquid scintillation counting.

Nuclear extracts and in vitro processing reactions. HeLa-S3 cell nuclear extracts were prepared as described by Moore and Sharp (15), using HeLa-S3 cells obtained as a cell pellet from the National Cell Culture Center. Some

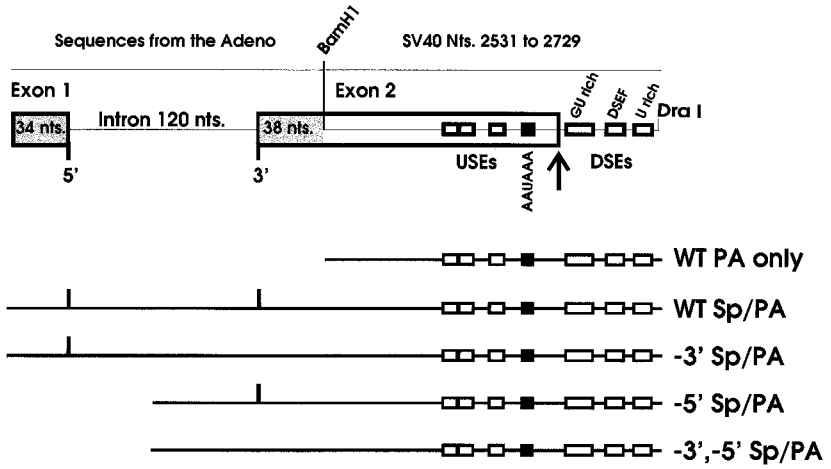

FIG. 2. Diagrams of RNA substrates used in in vitro cleavage and polyadenylation reactions. The diagram at the top shows the structure of the coupled splicing and polyadenylation template contained in pSP64-MXSVL (see Materials and Methods). The WT Sp/PA substrate was produced by in vitro transcription from pSP64-MXSVL. The other substrates shown were produced from various constructions made from pSP64-MXSVL and pGem-UPAS (see Materials and Methods). The templates are based on the SV40 late polyadenylation signal (SV40 nucleotides [Nts.] 2531 to 2729; *Bam*HI to *Dra*I; diagrammed on the top left). This signal contains, in addition to the AAUAAA and the cleavage-polyadenylation site (An), upstream sequence elements (USEs) and downstream sequence elements (DSEs) which affect the efficiency of polyadenylation (see references in the text). The WT PA only substrate was used to test polyadenylation since it contains only the SV40 late polyadenylation sequences and no splice sites. The substrate made to examine polyadenylation in the presence of splicing (WT Sp/PA) is the MXSVL substrate (diagrammed at the top) which includes a splicing cassette from the adenovirus (Adeno) major late region coupled to the SV40 late polyadenylation signal (21). The -3′ Sp/PA substrate contains a point mutation in the 3′ splice site making it nonfunctional for splicing (21). The -5′ Sp/PA substrate lacks the 5′ splice site as a result of deletion, and the -5′, -3′ Sp/PA substrate contains the 5′ splice site deletion and the 3′ splice site point mutation. See Materials and Methods for details of the construction of each mutant. Each substrate was prepared by in vitro transcription under conditions such that they were primed by a normal m^7GpppG cap or an alternate cap, either GMP-PCP or ApppG (see Materials and Methods).

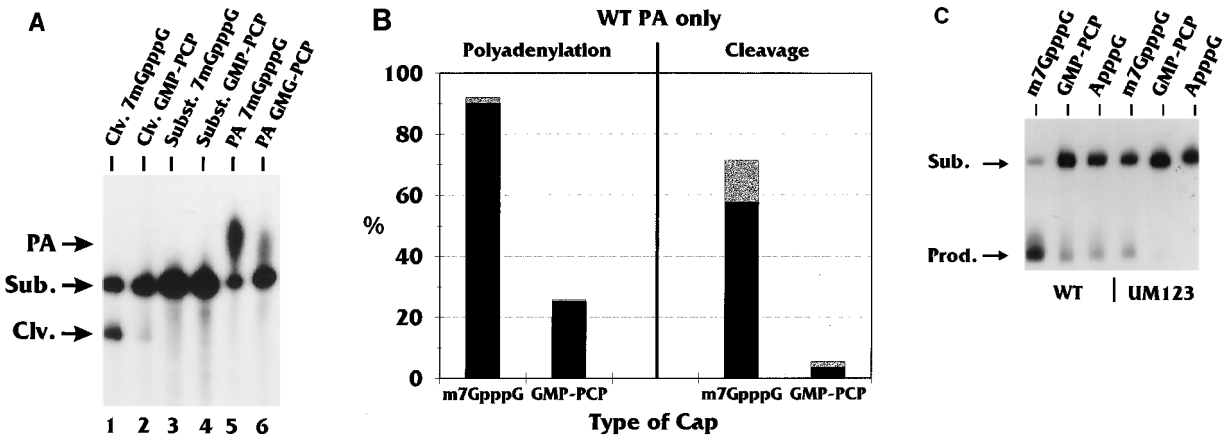

FIG. 3. The effect of cap on cleavage and polyadenylation reactions using the WT PA only substrate. (A) The WT PA only substrate was prepared with the m^7GpppG or GMP-PCP cap. Unreacted substrate (Subst.) containing each cap is shown in lanes 3 and 4. Each substrate was then subjected to the cleavage (Clv.) reaction in the presence of cordycepin (lanes 1 and 2) or to the full polyadenylation (PA) reaction (lanes 5 and 6). The reaction conditions and the electrophoresis conditions are described in Materials and Methods. (B) Quantitation of the cleavage and polyadenylation reactions using the WT Sp/PA substrate. The results of three separate reactions similar to those shown in panel A were quantitated with a Molecular Dynamics PhosphorImager, and the percent cleavage or polyadenylation was calculated. Black bars indicate averages, and gray bars indicate standard deviations. (C) Cleavage reactions were similar to those in panel A except that ApppG was used in addition to m^7GpppG and GMP-PCP. For the first three lanes (WT), the WT PA only substrate was used; for the last three lanes (UM123), the same substrate with the three upstream elements mutated by linker substitution (see text for details) was used.

variation in cleavage efficiency was noted between different nuclear extract preparations used in these studies. In vitro polyadenylation reaction mixtures contained (final concentrations) 60% (vol/vol) nuclear extract, 1 mM ATP (Pharmacia), 20 mM phosphocreatine (Sigma), 2.6% polyvinyl alcohol, and approximately 5 fmol of ^{32}P-labeled precursor RNA. Conditions for the in vitro cleavage reaction were the same except that 1 mM cordycepin 5′-triphosphate (Sigma) was used and the ATP concentration was reduced to 250 μM. Conditions for coupled splicing-polyadenylation reactions were the same as those of the cleavage reactions except that magnesium chloride was added to a final concentration of 1.5 mM (21). For competition analysis using cap analogs, exogenous cap analog was added to the in vitro reaction mixtures to give the indicated final concentrations (see Results) prior to initiation of the reaction by the addition of nuclear extract. Unless otherwise specified, processing reactions were performed at 30°C for 60 min and stopped by the addition of 8 volumes of 20 mM Tris (pH 7.5)–400 mM NaCl–0.01% sodium dodecyl sulfate. Reaction products were extracted once with phenol-chloroform-isoamyl alcohol, ethanol precipitated, and analyzed on 5% polyacrylamide–7 M urea gels. Substrate RNAs and processed species were quantitated with a Molecular Dynamics PhosphorImager. Percent processed RNA was calculated as the amount of processed species divided by the amount of unprocessed plus processed RNA.

RESULTS

RNA substrates used in in vitro processing reactions. In the following experiments, we used in vitro RNA processing reactions to test the effects of cap structures and splice sites in RNA processing, particularly polyadenylation. The RNA substrates prepared for these experiments are listed in Fig. 2. They are based on the SV40 late polyadenylation signal (SV40 nucleotides 2531 to 2729; *Bam*HI to *Dra*I). This signal contains, in addition to the AAUAAA and the cleavage-polyadenylation site, upstream elements and downstream elements which affect the efficiency of polyadenylation (3, 5, 24, 25, 26, 30). The substrate made to test polyadenylation only (WT PA only) contains only the SV40 late polyadenylation sequences and no splice sites. The substrate made to examine polyadenylation in the presence of splicing (WT Sp/PA) is the MXSVL substrate (diagrammed at the top of Fig. 2) which includes a splicing cassette from the adenovirus major late region coupled to the SV40 late polyadenylation signal (19, 21). Substrates made with specific splice sites deleted or mutated (-3′ Sp/PA, -5′ Sp/PA, and -5′,-3′ Sp/PA) were derivatives of MXSVL (see Materials and Methods). Each of these substrates was prepared by in vitro transcription under conditions such that they were primed by a normal m^7GpppG cap or an alternate cap, either GMP-PCP or ApppG (6, 9).

The cap structure affects the efficiency of cleavage and polyadenylation. The WT PA only substrate (Fig. 2) was prepared with the normal m^7GpppG cap as well as the cap analog GMP-PCP. Both RNAs were then tested in normal in vitro polyadenylation reactions as well as in cleavage reactions which contained cordycepin to inhibit polyadenylation so that the cleaved product is detectable. Figure 3A shows that the substrate with the m^7GpppG cap was efficiently cleaved and polyadenylated, whereas polyadenylation and cleavage of the substrate with a cap analog, GMP-PCP, were dramatically decreased. Figure 3B shows the quantitation of the results of three separate experiments to demonstrate the effect of the cap structure on the cleavage and polyadenylation of the WT PA only substrate. Cleavage was decreased at least 10-fold, whereas polyadenylation was reduced approximately 4-fold. The reduced effect on polyadenylation compared with cleavage is due to nonspecific polyadenylation of 3′ ends which occurs in these extracts. For this reason, in the following experiments our quantitative analyses rely on the results of cleavage reactions done in the presence of cordycepin.

In Fig. 3C, the WT PA only substrate was again prepared with m^7GpppG and GMP-PCP as well as a second cap analog, ApppG, and tested in the cleavage reaction. Again, the substrate with the m^7GpppG cap was efficiently cleaved whereas the cleavage of substrates containing either the GMP-PCP or ApppG cap was reduced at least 10-fold. Hence, the effect of cap analogs is seen with at least two different analogs.

Also shown in Fig. 3C are the results of similar experiments done with the PA-only substrate in which the three upstream element motifs of the SV40 late polyadenylation signal (Fig. 2) were mutated by linker substitution (UM123 [26]). The mutation of the upstream element motifs has been shown to dramatically affect the efficiency of polyadenylation of the substrate (26). This is clearly seen in Fig. 3C for the UM123 mutant substrate with an m^7GpppG cap. The combination of alternate caps (GMP-PCP or ApppG) and the upstream element mutations essentially eliminated the ability of the substrate to be cleaved.

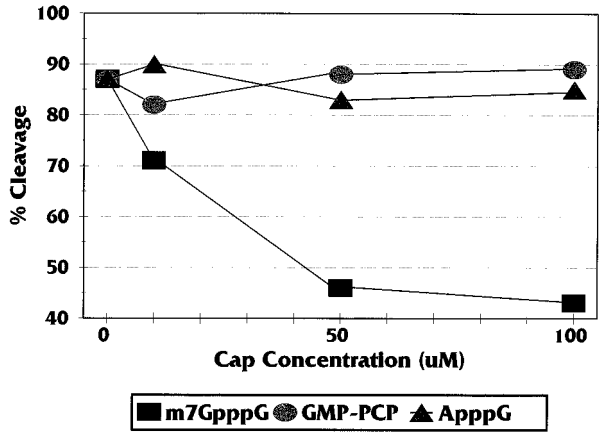

FIG. 4. Inhibition of cleavage by free m^7GpppG cap structure. The percent cleavage of the m^7GpppG-capped PA only substrate was determined in the presence of increasing concentrations of free m^7GpppG, GMP-PCP, or ApppG cap structure added in the indicated concentrations to the reaction mixtures.

Free m^7GpppG cap structure can inhibit cleavage. In Fig. 4, the percent cleavage of the m^7GpppG capped PA-only substrate was determined in the presence of increasing concentrations of free m^7GpppG, GMP-PCP, and ApppG cap structures added to the in vitro polyadenylation reaction mixtures. Only the m^7GpppG cap structure inhibited cleavage, indicating that titratable factors which interact with authentic cap structures, but not with cap analogs, are required for the efficient cleavage of the PA-only substrate.

Effects of cap structures on splicing and polyadenylation substrates. In Fig. 5A, the WT Sp/PA substrate (Fig. 2) was analyzed in a coupled in vitro splicing and polyadenylation reaction in which the substrate contained either an m^7GpppG cap or a GMP-PCP cap. With the m^7GpppG cap, the normal processing products can be seen when the reaction is done in the presence of cordycepin. These products (second lane in Fig. 5A) include the unspliced polyadenylated product (PA only), the spliced and unpolyadenylated product (Sp only), and the spliced and polyadenylated product (Sp/PA). When the same reaction is done with the substrate capped with GMP-PCP, several interesting observations are made. First, there are few spliced products, which agrees with the data of Izaurralde et al. (9) showing that an m^7GpppG cap and a nuclear CBC are essential for efficient in vitro removal of the adjacent intron in a simple one-intron substrate. Second, the levels of the total polyadenylated products (polyadenylated only plus spliced and polyadenylated) are very similar regardless of the cap used. This is shown graphically in Fig. 5B, where the quantitation of each product from three separate experiments are averaged and plotted for comparison. Clearly the alternate cap dramatically affected splicing of this substrate. However, in contrast to the PA-only substrate, the alternatively capped WT Sp/PA substrate is not dramatically decreased in total polyadenylation, suggesting that an element of the splice cassette can insulate polyadenylation from the need for an m^7GpppG cap.

The 3′ splice site insulates polyadenylation from effects of the cap. To determine why the alternative cap structure did not affect polyadenylation in the WT Sp/PA substrate, we tested the three mutant Sp/PA substrates shown in Fig. 2. All of these substrates are mutated in one or both of their splice sites and hence are similar to the WT PA only substrate in that they are capable of polyadenylation only. The -3′ Sp/PA substrate has a single point mutation in the 3′ splice site rendering it incapable

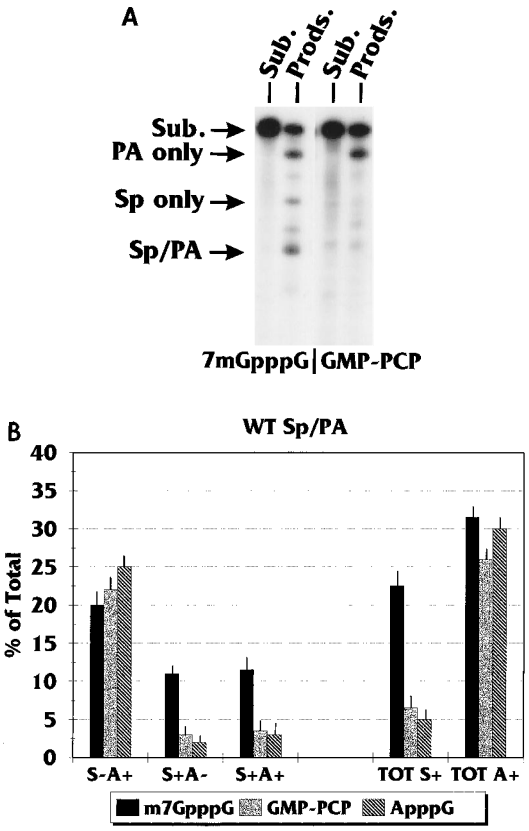

FIG. 5. The 3′ splice site replaces the need for the m^7GpppG cap. (A) The WT Sp/PA substrate capped with either m^7GpppG or GMP-PCP was analyzed in the coupled splicing and polyadenylation reaction. The migration of the unreacted substrate (Sub.) is indicated along with the various reaction products (Prods.): PA only, the product which is polyadenylated but not spliced; Sp only, the product which is spliced but not polyadenylated; and Sp/PA, the spliced and polyadenylated product. (B) Quantitation of the data of three separate experiments similar to that shown in panel A. Each reaction product was quantitated with a Molecular Dynamics PhosphorImager, and the percentage of the total was determined. The bars indicate averages, and the thin lines indicate standard deviations. Total spliced products (TOT S+) is the sum of the spliced-only product (S+A−) and the spliced and polyadenylated product (S+A+). Total polyadenylated products (TOT A+) is the sum of the polyadenylated-only product (S−A+) and the spliced and polyadenylated product (S+A+).

of splicing; however, the 5′ splice site remains intact (19, 21). Substrate -5′ Sp/PA is likewise rendered incapable of splicing through the deletion of the 5′ splice site; however, the 3′ splice site remains intact. In substrate -3′, -5′ Sp/PA, both mutations were combined to remove the 5′ and 3′ splice sites.

Figure 6 compares the quantitative results of three separate cleavage reactions using the -5′ Sp/PA and -3′, -5′ Sp/PA substrates with m^7GpppG and GMP-PCP caps. The presence of the cap analog had no significant effect on the percent cleavage of the -5′ Sp/PA substrate; however, the additional mutation of the 3′ splice site in the -3′/-5′ Sp/PA substrate caused inhibition of cleavage in the presence of the cap analog. These data indicate that the presence of a 3′ splice site provides a level of stimulation to polyadenylation comparable to that provided by an m^7GpppG cap and functionally replaces the need for the m^7GpppG cap.

A 3′ splice site allows efficient polyadenylation in the presence of free m^7GpppG cap structure. In Fig. 4, we showed that free m^7GpppG cap added to a reaction mix inhibited polyadenylation of the m^7GpppG-capped PA-only substrate as a result of the titration of m^7GpppG cap-binding factors. If a 3′

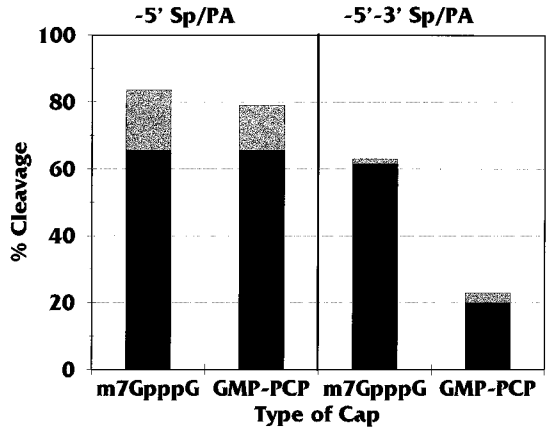

FIG. 6. Quantitative results of three separate cleavage reactions using the -5′ SP/PA and -3′,-5′ SP/PA substrates with m^7GpppG and GMP-PCP caps. The data were quantitated with a Molecular Dynamics PhosphorImager, and the percent cleavage was calculated. Black bars indicate averages, and gray bars indicate standard deviations.

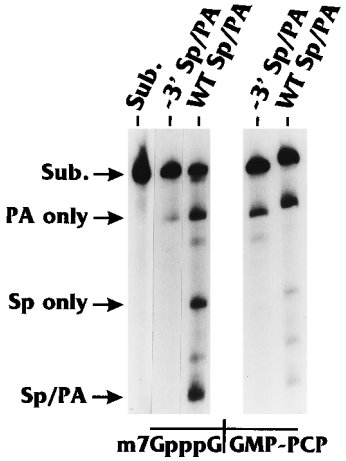

FIG. 8. The processing of the -3′ Sp/PA and WT Sp/PA substrates containing either the m^7GpppG or GMP-PCP cap was determined in the splicing and polyadenylation reaction. Abbreviations are as defined in the legend to Fig. 5A.

splice site can functionally replace the cap in affecting polyadenylation efficiency, then the 3′ splice site should also insulate polyadenylation from the effects of titration of the m^7GpppG cap-binding factors. In Fig. 7, we show that this is the case. The cleavage of the m^7GpppG-capped -5′ Sp/PA substrate was not affected by increased amounts of free m^7GpppG cap structure. However, the removal of the 3′ splice site by point mutation in the -3′,-5′ Sp/PA substrate again allowed the inhibition by free m^7GpppG cap structure.

A 5′ splice site upstream of the polyadenylation signal in conjunction with an m^7GpppG cap is inhibitory to polyadenylation. In Fig. 8, the processing of the -3′ Sp/PA substrate (Fig. 2) is compared with that of the WT Sp/PA substrate. In agreement with the data in Fig. 5A, the WT Sp/PA substrate capped with m^7GpppG forms all of the spliced and polyadenylated products, whereas the -3′ Sp/PA substrate, containing an intact 5′ splice site and mutated 3′ splice site, is neither spliced nor polyadenylated appreciably. This latter finding contrasts sharply with the other data for splicing-defective substrates in that the normal cap did not compensate for the absence of the 3′ splice site. This finding suggests that the presence of the 5′ splice site may be inhibitory.

The use of the GMP-PCP cap with these same substrates provided more insight into this effect of the 5′ splice site. In these experiments, we see that the WT Sp/PA substrate is not spliced but is highly polyadenylated as shown in Fig. 5A. However, using the -3′ Sp/PA substrate with the cap analog provides the very interesting result that about 25% of the wild-type level of cleavage is retained. This is the only condition in which a cap analog allowed more cleavage than the m^7GpppG cap. This result was consistently seen in repeated experiments using several extracts. However, it must be pointed out that the degree of the effect varied (10 to 25% of the wild-type level) with the extract, suggesting that a labile factor may be involved. Overall, these findings suggest that, at least in part, the negative effects of the 5′ splice site on cleavage occur in conjunction with the m^7GpppG cap.

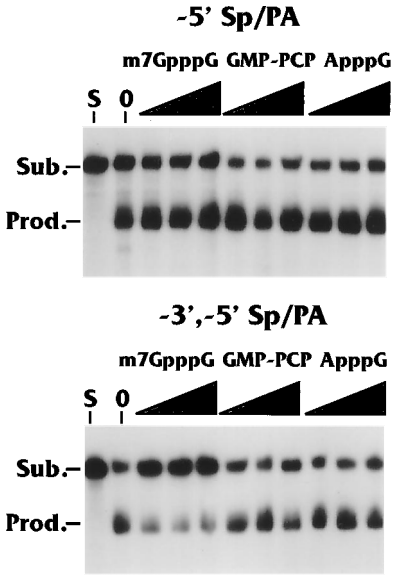

FIG. 7. Cleavage of the -5′ Sp/PA and -5′/3′ Sp/PA substrates capped with m^7GpppG was performed in the presence of increasing concentrations of free m^7GpppG cap structure or the cap analogs GMP-PCP and ApppG. The final concentrations of free cap added to the reaction were 10, 50, and 100 μM, respectively. Lane S shows the migration of the substrate (Sub.) alone; lane 0 shows the normal cleavage reaction with no added free cap structure. Prod., product.

DISCUSSION

Previous data (9) have suggested that a specific CBC (see the introduction), in association with the m^7GpppG cap, interacts with components of the splicing commitment complex at the 5′ splice site of the first exon (Fig. 1). This interaction provides a means to define the exon, or to activate the progression to full spliceosome assembly, thus positively affecting the removal of the first intron (9, 22). We have shown that the m^7GpppG cap can also positively affect polyadenylation of a PA-only substrate and that cap analogs do not do this because they fail to efficiently bind titratable cap-binding factors.

The low efficiency of polyadenylation seen in the presence of cap analogs can be restored by the presence of a 3′ splice site upstream of the polyadenylation signal. This finding is in agreement with the concepts of exon definition (Fig. 1), which suggests that splicing components at the 3′ splice site and components of the polyadenylation complex may interact to define the last exon. The fact that the cap and the 3′ splice site

are functionally interchangeable as affectors of polyadenylation suggests that they may utilize similar factors for completing the interactions between the ends of the exons. For example, the specific cap-binding proteins and the splicing components at the 3′ splice site may interact with similar mediating factors (possibly SR proteins or snRNPs, for example) which complete the interactions with the polyadenylation complex and increase polyadenylation efficiency. Similarly, these same mediating factors may function with splicing components at the 3′ splice site during the definition and processing of interior exons (Fig. 1).

Previous data have suggested that a 5′ splice site located upstream of a polyadenylation signal may be inhibitory. For example, Niwa et al. (20) suggested that the insertion of a 5′ splice site in a 3′-terminal exon is inhibitory to polyadenylation. Similarly, Wassarman and Steitz (28) have characterized a site upstream of the SV40 late polyadenylation signal which has weak homology to a 5′ splice site and weak interaction with U1 snRNP. When this site was changed to a consensus 5′ splice site, processing of the last exon was inhibited. In addition, a 5′ splice site in the 3′ untranslated region of the bovine papillomavirus type 1 late genes inhibits late gene expression apparently through the inhibition of polyadenylation of late mRNAs (7). In agreement with these data, we have found that the presence of a 5′ splice site located upstream of the AAUAAA of the SV40 late polyadenylation signal is inhibitory to polyadenylation. However, this inhibition requires, at least in part, an m^7GpppG cap, since some of the inhibition is relieved when a cap analog is used. This finding suggests that the interaction between the CBC at the cap and splicing components at the 5′ splice site forms a complex which is inhibitory to further RNA processing (at least polyadenylation) if continued processing is not possible. Such a situation would result from the lack of an upstream 3′ splice site and, therefore, the inability to remove an intron.

ACKNOWLEDGMENTS

We thank Susan Berget for providing plasmids and helpful discussion during the course of this work, Nancy Schek for experimental expertise, and Carol Lutz and Holly Hans for helpful comments on the manuscript. We also thank all members of the Alwine laboratory for helpful discussion, advice, and encouragement.

This work was supported by Public Health Service grant GM45773 awarded to J.C.A.

REFERENCES

1. **Ausubel, F. A., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.
2. **Berget, S. M.** 1995. Exon recognition in vertebrate splicing. J. Biol. Chem. **270:**2411–2414.
3. **Carswell, S., and J. C. Alwine.** 1989. Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. Mol. Cell. Biol. **9:**4248–4258.
4. **Chiou, H. C., C. Dabrowski, and J. C. Alwine.** 1991. Simian virus 40 late mRNA leader sequences involved in augmenting mRNA accumulation via multiple mechanisms, including increased polyadenylation efficiency. J. Virol. **65:**6677–6685.
5. **Conway, L., and M. Wickens.** 1985. A sequence downstream of AAUAAA is required for formation of simian virus 40 late mRNA 3′ termini in frog oocytes. Proc. Natl. Acad. Sci. USA **82:**3949–3953.
6. **Edery, I., and N. Sonenberg.** 1987. Cap-dependent RNA splicing in Hela nuclear extracts. Proc. Natl. Acad. Sci. USA **82:**7590–7594.
7. **Furth, P. A., W.-T. Choe, J. H. Rex, J. C. Byrne, and C. C. Baker.** 1994. Sequences homologous to 5′ splice sites are required for the inhibitory activity of papillomavirus late 3′ untranslated regions. Mol. Cell. Biol. **14:**5278–5289.
8. **Hashimoto, C., and J. A. Steitz.** 1986. A small nuclear ribonucleoprotein associates with the AAUAAA polyadenylation signal in vitro. Cell **45:**581–591.
9. **Izaurralde, E., J. Lewis, C. McGuigan, M. Jankowska, E. Darzynkiewicz, and I. W. Mattaj.** 1994. A nuclear cap binding complex involved in pre-mRNA splicing. Cell **78:**657–668.
10. **Luhrmann, R., B. Kastner, and M. Bach.** 1990. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. Biochem. Biophys. Acta **1087:**265–292.
11. **Lutz, C. S., and J. C. Alwine.** 1994. Direct interaction of the U1 snRNP-A protein with the upstream efficiency element of the SV40 late polyadenylation signal. Genes Dev. **8:**576–586.
12. **Lutz, C. S., K. G. K. Murthy, N. Schek, J. P. O'Connor, J. L. Manley, and J. C. Alwine.** 1996. Interaction between the U1 snRNP-A protein and the 160kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency *in vitro*. Genes Dev. **10:**325–337.
13. **Manley, J. L.** 1995. A complex protein assembly catalyzes polyadenylation of mRNA precursors. Curr. Opin. Genet. Dev. **5:**222–228.
14. **Moore, C. L., and P. A. Sharp.** 1984. Site-specific polyadenylation in a cell-free reaction. Cell **36:**581–591.
15. **Moore, C. L., and P. A. Sharp.** 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. Cell **41:**845–855.
16. **Moore, M. J., C. C. Query, and P. A. Sharp.** 1993. Splicing of precursors to messenger RNAs by the spliceosome, p. 303–358. *In* R. F. Gesteland and J. F. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
17. **Nesic, D., J. Cheng, and L. E. Maquat.** 1993. Sequences within the last intron function in RNA 3′ end formation in cultured cells. Mol. Cell. Biol. **13:**3359–3369.
18. **Nesic, D., and L. E. Maquat.** 1994. Upstream introns influence the efficiency of final intron removal and RNA 3′-end formation. Genes Dev. **8:**363–375.
19. **Niwa, M., and S. M. Berget.** 1991. Mutation of the AAUAAA polyadenylation signal depresses in vitro splicing of proximal but not distal introns. Genes Dev. **5:**2086–2095.
20. **Niwa, M., C. C. MacDonald, and S. M. Berget.** 1990. Are vertebrate exons scanned during splice site selection? Nature (London) **360:**277–280.
21. **Niwa, M., S. D. Rose, and S. M. Berget.** 1990. In vitro polyadenylation is stimulated by the presence of an upstream intron. Genes Dev. **4:**1552–1559.
22. **Ohno, M., H. Sakamoto, and Y. Shimure.** 1987. Preferential excision of the 5′ proximal intron from mRNA precursors with two introns as mediated by the cap structure. Proc. Natl. Acad. Sci. USA **84:**5187–5191.
23. **Raju, V. S., and S. T. Jacob.** 1988. Association of poly(A) polymerase with U1 RNA. J. Biol. Chem. **263:**11067–11070.
24. **Sadofsky, M., and J. C. Alwine.** 1984. Sequences on the 3′ side of hexanucleotide AAUAAA affect efficiency of cleavage at the polyadenylation site. Mol. Cell. Biol. **4:**1460–1468.
25. **Sadofsky, M., S. Connelly, J. L. Manley, and J. C. Alwine.** 1985. Identification of a sequence element on the 3′ side of AAUAAA which is necessary for simian virus 40 late mRNA 3′ end processing. Mol. Cell. Biol. **5:**2713–2719.
26. **Schek, N., C. Cooke, and J. C. Alwine.** 1992. Definition of the upstream efficiency element of the simian virus 40 late polyadenylation signal by using in vitro analyses. Mol. Cell. Biol. **12:**5386–5393.
27. **Wahle, E., and W. Keller.** 1992. The biochemistry of 3′-end cleavage and polyadenylation of messenger RNA precursors. Annu. Rev. Biochem. **61:**419–440.
28. **Wassarman, K. M., and J. A. Steitz.** 1993. Association with terminal exons in pre-mRNAs: a new role for the U1 snRNP? Genes Dev. **7:**647–659.
29. **Wickens, M.** 1990. How the messenger got its tail: addition of poly(A) in the nucleus. Trends Biochem. Sci. **15:**277–281.
30. **Wilusz, J., and T. Shenk.** 1990. A uridylate tract mediates efficient heterogeneous nuclear ribonucleoprotein C protein-RNA cross-linking and functionally substitutes for the downstream element of the polyadenylation signal. Mol. Cell. Biol. **10:**6397–6407.