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Inhibition of splicing but not cleavage at the 5′ splice site by truncating human β-globin pre-mRNA

(ribonucleoproteins/lariat formation)

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ABSTRACT **Human β-globin mRNAs truncated in the second exon or in the first intron have been processed *in vitro* in a HeLa cell nuclear extract. Transcripts containing a fragment of the second exon as short as 53 nucleotides are efficiently spliced, whereas transcripts truncated 24 or 14 nucleotides downstream from the 3′ splice site are spliced inefficiently, if at all. All of these transcripts, however, are efficiently and accurately cleaved at the 5′ splice site. In contrast, RNA truncated in the first intron, 54 nucleotides upstream from the 3′ splice site, is not processed at all. These findings suggest that cleavage at the 5′ splice site and subsequent splicing steps—i.e., cleavage at the 3′ splice site and exon ligation—need not be coupled. Anti-Sm serum inhibits the complete splicing reaction and cleavage at the 5′ splice site, suggesting involvement of certain ribonucleoprotein particles in the cleavage reaction. ATP and Mg^{2+} are required for cleavage at the 5′ splice site at concentrations similar to those for the complete splicing reaction.**

Recently, several systems for *in vitro* splicing of eukaryotic mRNA have been developed. The early systems relied either on coupled transcription and splicing of mRNA transcripts in whole cell extracts from HeLa cells (1–3) or on the addition of exogenous precursor mRNA to whole cell extracts from HeLa cells (4) or to nuclear extracts from MOPC-315 cells (5, 6). Progress was slow due to the inefficiency of the splicing extracts and the limited availability of substrate RNA. The recent development of a highly efficient nuclear splicing extract (7, 8) and the methods to transcribe large amounts of precursor RNA from DNA templates cloned under the SP6 promoter (9, 10) led to rapid advances in this field.

The steps in mRNA splicing, formulated by recent *in vitro* studies (refs. 11–16; see ref. 17 for a recent review), appear to be as follows: a capped RNA transcript is cleaved at the 5′ splice site and, simultaneously, the first nucleotide of the intron attacks an adenosine residue located 20–40 nucleotides (nt) upstream from the 3′ splice site, forming a lariat structure. This lariat intermediate is then cleaved at the 3′ splice site with concomitant ligation of the 5′ exon, thus generating a spliced mRNA and the free intron in the lariat form. The details of the phosphodiester bond formation during splicing have been determined (13) and the participation of U1 ribonucleoprotein (U1-RNP) in the splicing reaction has been convincingly shown (18–21).

In spite of this remarkable progress, many aspects of the splicing reaction are still poorly understood. The mechanisms of splice site selection and alternative splicing are unknown as are the properties of the partially purified splicing enzymes (22). The events that occur during a pronounced initial lag period that is observed *in vitro* (7) as well as the properties of and relationship among the subsequent splicing steps have not been analyzed in detail.

Although several sequence elements are known to be essential for splicing [splice site consensus sequences, polypyrimidine stretch, branch point (17)], it is not known if exon sequences play any role in splicing. It could be expected that exon sequences contribute to the overall structure of the precursor RNA that is necessary for splicing. We have addressed this problem by studying *in vitro* splicing of human β-globin mRNA precursor transcripts that have been systematically truncated within the second exon of the globin gene. The results reported here show that transcripts missing most but not all of the second exon sequences are not spliced but are only cleaved at the 5′ splice site.

MATERIALS AND METHODS

DNA templates were prepared by linearizing the plasmid pSP64HbΔ6 (7) with restriction enzymes that cleave within the second exon and the first intron of the human β-globin gene (see Fig. 1).

Transcription and capping of the substrate RNA was carried out essentially as described by Konarska *et al.* (14). *In vitro* splicing reactions were performed at 30°C for 2 hr in a 25-μl reaction mixture with 5 mM ATP, 3.3 mM $MgCl_2$, 20 mM creatine phosphate, 2.6% polyvinyl alcohol, 60% HeLa cell nuclear extract, and [^{32}P]RNA (7, 8). [^{32}P]RNA products from *in vitro* processing reactions were electrophoresed on a 5% polyacrylamide sequencing gel (23) and autoradiographed. For more detailed analysis, the products from 30 reaction mixtures were pooled and separated on several gels. The appropriate bands were eluted, digested with RNase T1 (5 units of the enzyme in a 5-μl reaction mixture in 10 mM Tris·HCl, pH 7.5/1 mM EDTA buffer) for 20 min at 37°C, and analyzed further on a 20% polyacrylamide sequencing gel. Alternatively, some RNAs were analyzed by a standard two-dimensional fingerprinting procedure (24).

Inhibition of the processing reactions was carried out by incubating 15 μl of the nuclear extract with 1 μl of a purified IgG fraction (5–15 mg of protein per ml) of sera from patients with systemic lupus erythematosus (SLE) for 30 min at 0°C. The IgG fractions were prepared as described (25) and dialyzed against 10 mM Tris·HCl, pH 7.5/10 mM NaCl. The pretreated extract was then used in the splicing reaction under standard conditions.

RESULTS

Processing of Human β-Globin mRNA Truncated in the Second Exon. We have used a HeLa cell nuclear splicing extract (7, 8) to study processing of a series of RNA transcripts terminating within the second exon of the human β-globin gene (Fig. 1). The longest transcript, terminating at the *Bam*HI site within the second exon, gave rise to four

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Abbreviations: RNP, ribonucleoprotein; snRNP, small nuclear RNP; SLE, systemic lupus erythematosus; nt, nucleotide(s).

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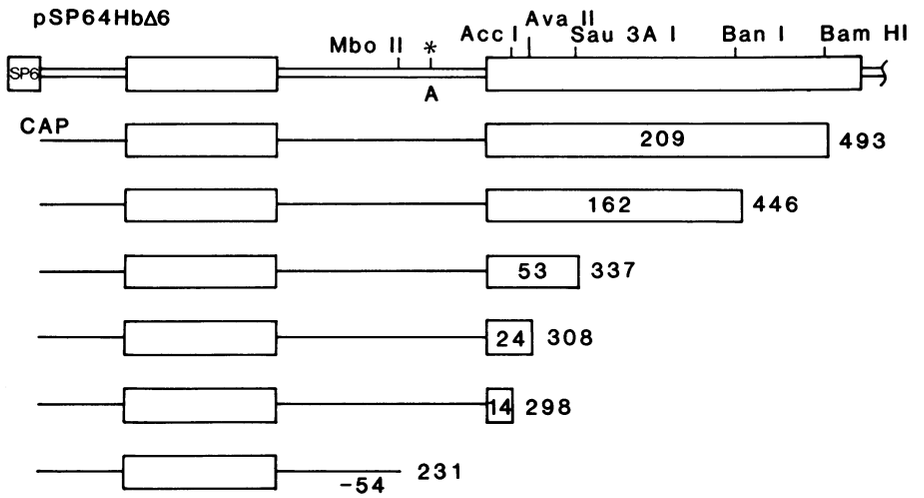


FIG. 1. Structure of the RNA transcripts and the DNA template from human β-globin gene. The top diagram represents a fragment of the DNA template, derived from a human β-globin gene cloned under the SP6 promoter (plasmid pSP64HbΔ6). The template was linearized with indicated restriction enzymes and used for *in vitro* transcription with SP6 polymerase. The transcribed RNAs, which are truncated at the appropriate restriction sites, are diagrammed below. Open boxes, exons; open box labeled SP6, SP6 promoter; thin lines, introns and untranslated region. The asterisk indicates the branch point of the lariat structure. The numbers next to the transcripts indicate nucleotide length of the transcript. The numbers inside the exons indicate the distance (in nucleotides) from the 3′ splice site to the 3′ end of the transcript. The distance from the 5′ end of the transcript to the 5′ splice site is 154 nt.

prominent products in addition to the unprocessed input RNA (Fig. 2, lane 1). Ruskin *et al.* (12) characterized these products and showed them to be the intron in the lariat form attached to the second exon, spliced mRNA, 5′-proximal sequences containing the 5′ exon, and the excised intron (Fig. 2, lane 1, bands b–e, respectively). We have analyzed all of the reaction products by digestion with RNase T1 and have found that the gel pattern of the digests was consistent with the expected sequences of the respective RNAs. Moreover, bands b and e exhibited aberrant mobility on polyacrylamide gels of different porosity, a feature characteristic of lariat structures (results not shown; see also Fig. 4).

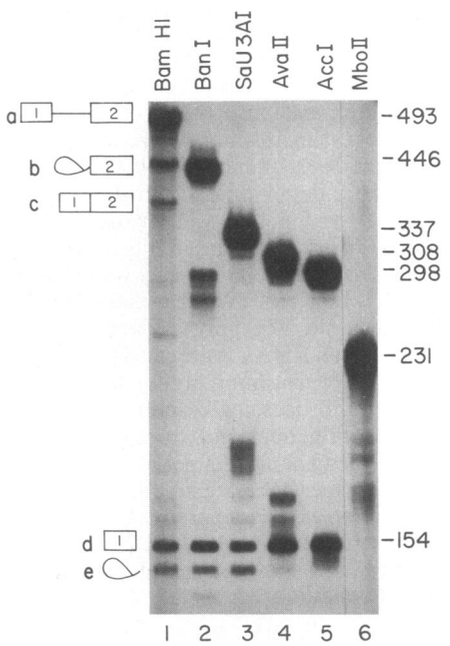


FIG. 2. *In vitro* splicing of the truncated human β-globin mRNA precursors. RNA transcribed and processed as described in the text was isolated and analyzed on a 5% polyacrylamide sequencing gel. Restriction enzymes used to linearize and truncate the DNA template are indicated at the top. The length in nucleotides of each of the transcripts and of the 5′ fragment is shown on the right. The structure of the RNA in bands a–e (lane 1) is indicated on the left. a, input RNA; b, 3′ half carrying the intron in the lariat form; c, the spliced product; d, 5′ fragment containing the first exon and 5′ untranslated sequence; e, released intron in the lariat form. Additional minor bands in lane 1 are most likely unspecific degradation products.

Lanes 2–5 in Fig. 2 show the results of processing RNA run-off products terminating 162 (*Ban* I), 53 (*Sau*3AI), 24 (*Ava* II), and 14 (*Acc* I) nt downstream from the 3′ splice site. These transcripts, when processed in the splicing extract, each gave rise to a band comigrating with the 5′ exon (band d), indicating that cleavage at the 5′ splice site occurred efficiently regardless of the length of the substrate. Transcripts in lanes 2 and 3 also generated band e as a prominent product, which has the mobility of the free intron in the lariat form when electrophoresed on the 5% (Fig. 2, lanes 1–3) and 8% (not shown) polyacrylamide sequencing gels. Quantitation of band e in each lane by scintillation spectroscopy indicates that this product is generated in comparable amounts during processing of all three transcripts. Since this product is diagnostic for the ligation step in the splicing reaction (11–17), it is likely that the transcripts truncated 162 and 53 nt downstream from the 3′ splice site are spliced as efficiently as the longest transcript. We have not analyzed the other bands that are seen in lanes 2 and 3 but their numbers and their mobilities on the gel suggest that they are the expected products and intermediates of splicing. First, there is a band in each lane that migrates as exon 1 (band d, lane 1). Second, there is a band whose mobility on the gel is consistent with the expected size of the spliced product (316 nt for *Ban* I and 207 nt for *Sau*3AI).

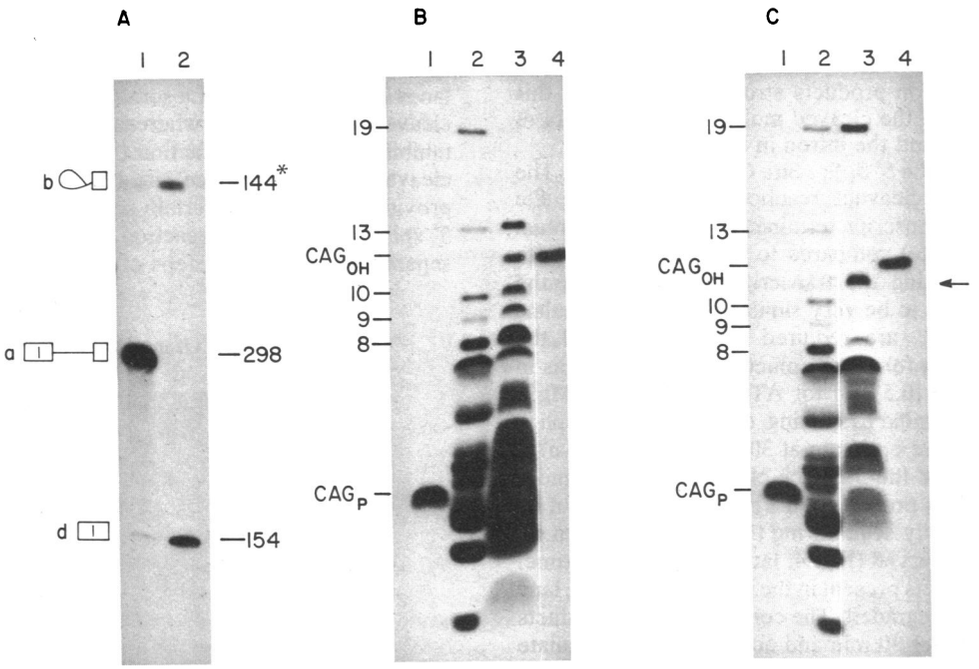
Transcripts terminating 24 and 14 nt downstream from the 3′ splice site were spliced very inefficiently, if at all. This is shown by the trace amounts or complete lack of the free intron, band e, in lanes 4 and 5 (Fig. 2). Although these transcripts are not effectively spliced, the band that comigrates with band d, lane 1, is produced in large amounts. The analysis of this band from lane 5 is shown below.

Fig. 2, lane 6, shows the results of processing a transcript that terminates within the intron, 54 nt upstream from the 3′ splice site. Under normal splicing conditions, this transcript was not processed at all. Since it lacks the 3′ splice site, the absence of a spliced product was expected. However, the absence of a band containing the 5′ exon (band d) indicates

that cleavage at the 5′ splice site was also prevented, suggesting that the RNA sequence missing in this transcript plays a role in cleavage at the 5′ splice site (see *Discussion*).

Analysis of the Cleavage Products. From the results presented in Fig. 2, lane 5, it appears that during processing of the transcript terminating 14 nt downstream from the 3′ splice site no spliced product is generated. The only product that is visible is a band comigrating with the 5′ exon. If this band is produced by cleavage at the 5′ splice site, one would also expect to see a 3′ half of the molecule containing the intron and 14 nt of the second exon. Since the 3′ half of the molecule could be in the lariat form (11, 12), it may have aberrant mobility and comigrate with the 5′ exon on the 5% polyacrylamide sequencing gel. To identify the RNA in this band, the material was eluted from the gel and electrophoresed on a 12% polyacrylamide sequencing gel. Under these conditions, the material separated into two bands, one with the mobility of the 5′ exon (Fig. 3*A*, lane 2, band d) and the other migrating slower than the input RNA (Fig. 3*A*, lane 2, band b). Since the RNA in the latter band has an unusual mobility, it is most likely the 3′ half of the molecule, consisting of the intron in the lariat form and the 14 nt of the second exon.

To analyze these products in more detail and to ascertain that the cleavage occurred exactly at the 5′ splice site, we have treated the RNAs from bands b and d (Fig. 3*A*, lane 2) with RNase T1 and analyzed the products of digestion on a 20% polyacrylamide sequencing gel. Analysis of band d (Fig. 3*B*, lane 3) showed that this RNA contained all of the large oligonucleotides characteristic of the 5′ exon (the 13-mer, a 10-mer, and the 9-mer; see Fig. 3*D*). In addition, a band was present that was not found in an RNase T1 digest of the unprocessed run-off transcript (Fig. 3*B*, lane 2) and that comigrated with the trinucleotide CAG (CAG_{OH}, Fig. 3*B*, lane 4). The RNase T1 digest of the unprocessed transcript generates CAGp, a unique trinucleotide located at the 5′ splice junction (see Fig. 3*D*). Therefore, CAG trinucleotide could be generated in an RNase T1 digest of band d only if cleavage during splicing of the precursor RNA occurred



D

Gppp GAAUACAAGCUUACAUUUGCUUCUGACACAACUGUGUUCACUAGCAACCUCAAACAGACACCAUGGUGCACCUGA

8 10 9 8 13 8

CUCCUGAGGAGAAGUCUGCCGUUACUGCCCUGUGGGGCAAGGUGAACGUGGAUGAAGUUGGUGGUGAGGCCCUGG

GCAGguugguaucaaggauacaagacaggauuuaaggagaccaauagaaacugggcauguggagacagagaagacu

8

cuugggauuucugauaggcacugacucucucugccuauuggucuauuuucccaccuuagGCUGCUGGUGGUCU

10 19

FIG. 3. Analysis of the products generated by cleavage at the 5′ splice site. (*A*) Analysis of the cleavage products on a 12% polyacrylamide gel. Lane 1, RNA transcribed from the *Acc* I truncated template and processed as above (see Fig. 2, lane 5). Lane 2, RNA isolated from band d (Fig. 2, lane 5), reanalyzed on 12% gel. The sizes of the input RNA and of the cleavage products are shown on the right. Asterisk indicates the abnormal mobility of the lariat-containing fragment. The structure of the products is shown on the left. The small open box represents 14 nt of the second exon. (*B*) Analysis of the fragment containing the 5′ exon by RNase T1 digestion. RNA eluted from band d (*A*, lane 2) was digested with RNase T1 and analyzed on a 20% polyacrylamide sequencing gel. For comparison, input RNA—i.e., the transcript truncated 14 nt downstream from the 3′ splice site—was treated in the same manner. CAGp, used as a marker, was isolated from the appropriate spot in the fingerprint of the input RNA. CAG (CAG_{OH}) was obtained by treatment of the CAGp trinucleotide with alkaline phosphatase. Lane 1, CAGp trinucleotide. Lane 2, RNase T1 digest of the input RNA (band a, *A*, lane 1). Lane 3, RNase T1 digest of the 5′ exon-containing fragment (band d, *A*, lane 2). Lane 4, CAG trinucleotide. Numbers indicate the lengths of the characteristic oligonucleotides. (*C*) Analysis of the 3′ half of the molecule that contains the intron in the lariat form. Material eluted from band b (*A*, lane 2) was analyzed as in *B*. Lane 1, CAGp. Lane 2, input RNA. Lane 3, 3′ half of the molecule; a new oligonucleotide not present in the input RNA is indicated by the arrow. Lane 4, CAG. (*D*) Sequence of the RNA transcribed from the *Acc* I truncated template. Untranslated and exon sequences are in uppercase letters; intron is in lowercase letters. Characteristic oligonucleotides that are indicated in *B* and *C* are underlined. The asterisk indicates the branch point of the lariat structure.

exactly at the 5′ splice site between the first guanosine and the 5′ phosphate group: ...GpGp*CpApG*/pGpUpUpGp.... Cleavage by splicing enzyme(s) at any other position would generate a trinucleotide CAGp, which migrates in a different position on the gel (Fig. 3*B*, lane 1). Therefore, it can be concluded that in the nuclear extract, the cleavage reaction that produced band d occurred exactly at the 5′ splice site, resulting in a 5′ exon with a hydroxyl group at its 3′ end, in agreement with similar observations by Padgett *et al.* (13).

Fig. 3*C* shows an analysis of the RNA eluted from band b (Fig. 3*A*). This RNA lacked all of the oligonucleotides discussed above but contained a unique 19-mer that is located within the intron adjacent to the 3′ splice site (Fig. 3*C*, lane 3). A 10-mer, which should be present if this RNA contained the intron, appeared to have the approximate mobility of an 11-mer. The altered mobility of the 10-mer is expected for an RNA containing the lariat structure before RNase T1 digestion because the 10-mer should contain the branch point, an additional guanosine residue attached by a 2′,5′-phosphodiester bond to the adenosine residue of the oligonucleotide (indicated by the asterisk in the sequence in Fig. 3*D*). The aberrant mobility of the RNA from band b on sequencing polyacrylamide gels of different porosities and the analysis of its RNase T1 digestion products strongly suggest that this RNA is the 3′ half of the cleaved molecule containing 14 nt of the second exon and the intron in the lariat form.

Characteristics of the 5′ Splice Site Cleavage Reaction. The requirements of the cleavage reaction at the 5′ splice site (using the run-off transcript terminating 14 nt downstream from the 3′ splice site) compared to those of the complete splicing reaction (using the transcript with 209 nt of the second exon) appear to be very similar (4, 7). In particular, both ATP and Mg^{2+} are required for cleavage and the concentration optima for these cofactors are the same as in the splicing reaction [0.5 mM for ATP and 3 mM for Mg^{2+} (data not shown)]. Similar to splicing, cleavage at the 5′ splice site has a temperature optimum at 30°C (data not shown).

The time course of the 5′ splice cleavage reaction under optimal conditions is presented in Fig. 4. A lag of about 30 min, which is normally seen during the splicing reaction (7, 11–15), was also observed (Fig. 4, lane 2). During this time, all breakdown products present in the input RNA (Fig. 4, lane 1) were completely degraded. The correct cleavage products started to appear after 30 min and continued to accumulate for 2 hr (Fig. 4, lanes 3–5). This result suggests that any aberrant RNA products are selectively destroyed by nucleases while the intermediates of the splicing reaction are protected from degradation. After 4 hr, a portion of the input RNA and the 5′ splice site cleavage products have been degraded in this experiment (Fig. 4, lane 6); however, in other experiments, the degradation at this time point was not pronounced.

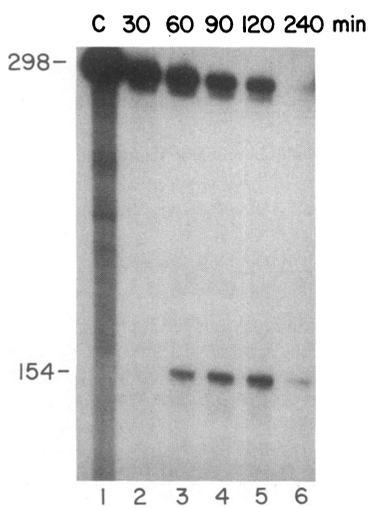


FIG. 4. Time course of cleavage at the 5′ splice site. RNA truncated 14 nt downstream from the 3′ splice site has been processed for the times indicated and analyzed as in Fig. 2. The lengths of the input RNA and of the 5′ exon-containing fragment are indicated. C, unprocessed input RNA.

Inhibition of Cleavage and Splicing by Antisera to Small Nuclear RNPs (snRNPs). Sera from patients with SLE, which contain autoantibodies with anti-Sm and anti-U1-RNP specificity, inhibit the splicing reaction (18, 20). These autoantibodies are directed against snRNP particles (26) and presumably block correct interactions of U1-RNP and/or other snRNPs with the rest of the splicing machinery and with the substrate RNA. There is evidence that U1-RNP binds to the 5′ splice site (27) but it is not known whether U1-RNP or other snRNPs play a role in cleavage at the 5′ splice site or in other steps of the splicing reaction. Fig. 5 shows the results of the inhibition of the splicing (Fig. 5, lanes 1–3) and the cleavage (Fig. 5, lanes 4–6) reactions using SLE antisera with anti-Sm specificity. Preincubation of the extract with normal (Fig. 5, lanes 2 and 5) and anti-La sera (not shown) had no effect on cleavage and splicing, whereas anti-Sm serum completely inhibited the splicing reaction (Fig. 5, lane 3) as well as the cleavage at the 5′ splice site (Fig. 5, lane 6). These data provide evidence that certain snRNAs are necessary for the 5′ splice site cleavage reaction, even when this reaction is separated from further steps of splicing.

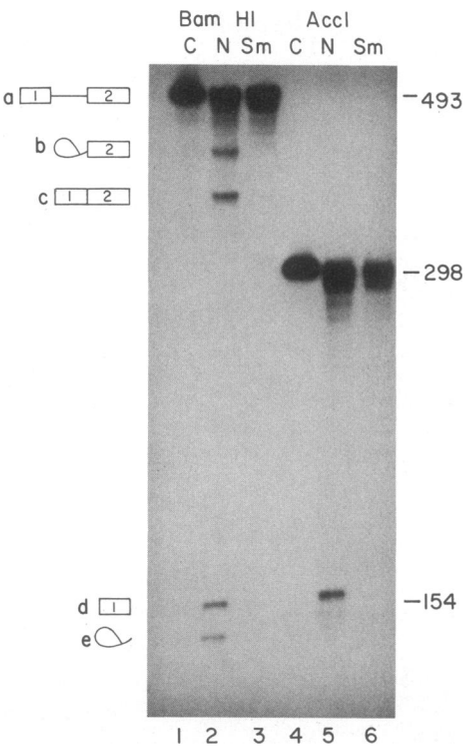


FIG. 5. Inhibition of RNA splicing and cleavage by the sera from SLE patients. The splicing extract was preincubated with the sera and pretreated extract was then used in the splicing reaction under standard conditions. RNAs transcribed from the template truncated with *Bam*HI (lanes 1–3) or *Acc* I (lanes 4–6) were used in this experiment. Extract was pretreated with the following: serum from a healthy individual (lanes 2 and 5) or anti-Sm serum (lanes 3 and 6). Control unprocessed RNAs are shown in lanes 1 and 4. C, control serum; N, normal serum. The lengths of the transcripts and of the 5′ exon are indicated on the right.

DISCUSSION

The experiments presented here describe several important features of the splicing mechanism. Human β-globin transcripts containing a fragment of the second exon as short as 53 nt were efficiently spliced in a nuclear extract, whereas transcripts truncated 24 or 14 nt downstream from the 3′ splice site were spliced inefficiently, if at all. All of these transcripts, however, appeared to be cleaved efficiently and accurately at the 5′ splice site. This was specifically shown for the transcript terminating 14 nt downstream from the 3′ splice site, which generated two products, one containing correctly terminated exon 1 and the other containing the 3′ half of the RNA carrying the intron in the lariat form. The lack of splicing of the shortest transcript suggests that either there is a minimum size requirement of the second exon for splicing or an important sequence found in this exon has been deleted in the truncated transcript. Since exon sequences differ from one another in size and primary sequence, it is very unlikely that the deletion of specific nucleotides is directly responsible for our results.

A transcript truncated 54 nt upstream from the 3′ splice site was not cleaved at the 5′ splice site, suggesting that 3′-proximal sequences are required for correct cleavage. Since this transcript lacks the 3′ splice site, the polypyrimidine stretch, and the lariat branch point, it appears that some or all of these sequences are required for 5′ cleavage. Reed and Maniatis (16) reported recently that cleavage at the 5′ splice site is blocked by deleting the polypyrimidine stretch and the 3′ splice site but not by mutating the AG dinucleotide in the 3′ splice site consensus sequence. Therefore, it appears that the polypyrimidine stretch plays an important role in the cleavage reaction.

It is interesting that the cofactor optima are the same for cleavage at the 5′ splice site and for the complete splicing reaction. Some of these cofactors may be required only during the initial step in splicing. It has been shown that ATP and Mg^{2+} are required during the lag period in the *in vitro* splicing reaction at the time when presumably a RNP complex is assembled (7). One can then speculate that the actual processing steps—i.e., cleavage, lariat formation, and final ligation—may proceed in an "activated" RNP complex without further involvement of the cofactors.

Previous experiments have shown that splicing can be inhibited *in vitro* by anti-U1-RNP and anti-Sm sera, indicating that snRNPs are involved in this process (18, 19). However, due to the limitations of the methods used in these experiments, it was not possible to differentiate between inhibition of cleavage at the 5′ splice site and inhibition of the subsequent steps of splicing. Results presented in Fig. 5 show that snRNPs are specifically required for the cleavage reaction at the 5′ splice site since anti-Sm IgG inhibited this step. This result is similar to the effects observed for splicing of simian virus 40 precursor mRNA *in vivo* in *Xenopus* oocytes (20).

Our results indicate that cleavage at the 5′ splice site can be carried out independent of 3′ splice site cleavage and exon ligation. Truncation of the substrate (shown above), mutation at the 3′ splice site (16), or heat treatment (45°C, 30 min) of the nuclear extract (unpublished data) blocks cleavage at the 3′ splice site and exon ligation without affecting the 5′ splice site cleavage step. These results suggest that cleavage at the 5′ splice site is carried out by a subset of the factors involved in splicing. Cleavage at the 5′ splice site seems to be quite complex, as suggested by the requirement for the distal downstream sequences in the intron, the concomitant formation of the lariat structure, and the requirement for snRNP components. However, the ability to separate cleavage at the 5′ splice site from further steps of mRNA splicing should simplify more detailed characterization of the splicing mechanism.

Note Added in Proof. Since this manuscript was submitted, Ruskin and Green (28) have reported that a human β-globin transcript truncated 14 nucleotides from the 3′ splice site is not spliced but is cleaved at the 5′ splice site.

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