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Short Donor Site Sequences Inserted within the Intron of β-Globin Pre-mRNA Serve for Splicing In Vitro

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We constructed SP6-human β-globin derivative plasmids that included possible donor site (5′ splice site) sequences at a specified position within the first intron. The runoff transcripts from these templates truncated in the second exon were examined for splicing in a nuclear extract from HeLa cells. In addition to the products from the authentic donor site, a corresponding set of novel products from the inserted, alternative donor site was generated. Thus, a short sequence inserted within an intron can be an active donor site signal in the presence of an authentic donor site. The active donor site sequences included a 9-nucleotide consensus sequence, 14- or 16-nucleotide sequences at the human β-globin first or second donor, and those at simian virus 40 large T antigen or small t antigen donor. These included 3 to 8 nucleotides of an exon and 6 to 8 nucleotides of an intron. The activity of the inserted donor site relative to that of the authentic donor site depended on the donor sequence inserted. The relative activity also strongly depended on the concentrations of both KCl (40 to 100 mM) and $MgCl_2$ (1.6 to 6.4 mM). At the higher KCl concentrations tested, all the inserted, or proximate, donor sites were more efficiently used. Under several conditions, some inserted donor sites were more active than was the authentic donor site. Our system provides an in vitro assay for donor site activity of a sequence to be tested.

Pre-mRNA splicing is a process that demands strict accuracy, because even a 1-nucleotide (nt) error in the splicing will result in an RNA that does not encode a functional polypeptide (for reviews, see references 10 and 25). It is obvious that some sequence elements of the pre-mRNA around the splice sites (22) and branch point (29) are important determinants of accurate splice site selection. However, these sequence elements may not always account for the accurate splicing, since many sequences resembling those around the splice sites are present in the pre-mRNA but are not used (24). The mechanism of this selection is also important for alternative splicing that gives rise to plural mRNA species from a single transcription unit as a result of choice among plural splice sites (for reviews, see references 3 and 18). To obtain information on splice site selection, assays for *cis* competition between splice sites have heretofore been used. Lang and Spritz (16) and Kühne et al. (15) tandemly duplicated the splice sites of human γ-globin and rabbit β-globin genes, respectively, and tested their utilization for splicing in vivo. In the former report, upstream splice sites were used, while external splice sites were used in the latter. Similarly, but more extensively, Reed and Maniatis (30) examined relative use of tandemly duplicated splice sites in human β-globin transcripts in vitro. They demonstrated that exon sequences, as well as proximity of donor and acceptor sites, play an important role in splice site selection in their system. Furthermore, the apparently conflicting results of Lang and Spritz and those of Kühne et al. could be reconciled in the light of the results obtained by Reed and Maniatis.

On the other hand, experiments including selection between different splice sites, as seen in the splicing of a natural pre-mRNA, have also been reported. Fu and Manley (9) examined the splice site selection in vivo of simian virus 40 (SV40) pre-mRNA derivatives from an alternatively spliced one. They showed that donor site inserts carrying 21- or 11-nt exon sequences were efficiently used, and that intron size, splice site position, and splice site sequence influence the choice of the splice site. Eperon et al. (8) found that 9-nt sequences of various donor sites, which were inserted upstream of an authentic donor site in the rabbit β-globin transcript, were used preferentially in vivo. Thus, in the selection between different splice sites, the relative contribution of sequences outside of a splice site sequence seemed to be less clear and possibly different from that in the selection between identical sites. We report here the effective utilization in vitro of a short donor site sequence inserted within an intron and factors influencing its activity.

MATERIALS AND METHODS

Construction of plasmids. The human β-globin gene carried in plasmid pSP64-HβΔ6 was kindly provided by T. Maniatis (13). The *Hin*dIII-*Bam*HI fragment carrying the first exon, the first intron, and a major part of the second exon was recloned between the *Hin*dIII and *Bam*HI sites of pSP64 (21). All the constructions were based on this plasmid. To introduce a unique restriction site for fragment insertion, the plasmid was cleaved by *Nsp*(7524)I at the four sites, including one 54 base pairs (bp) downstream of the first donor site. The fragments were blunt ended by mung bean nuclease and the DNA polymerase I Klenow fragment, ligated with an 8-bp *Xba*I linker, and digested with *Xba*I, *Hin*dIII, and *Eco*RI. The *Hin*dIII-*Xba*I 207-bp fragment and the *Xba*I-*Eco*RI 309-bp fragment were isolated for recloning into pSP64. The obtained plasmid pSP64-HβUX2 has a unique *Xba*I site 50 bp downstream from the authentic first donor site (4 bp to the 5′ side of the linker and 1 bp to the 3′ side were deleted during construction). Short DNA fragments carrying various donor-related sequences were derived from the pSP64 derivative plasmids described previously (37). Following *Hin*dIII cleavage, the plasmid DNAs were blunt ended by the Klenow fragment, ligated with the *Xba*I linker,

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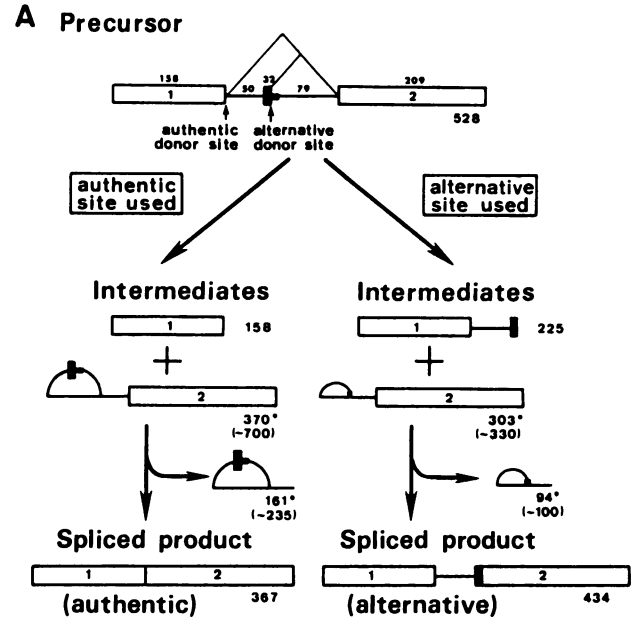

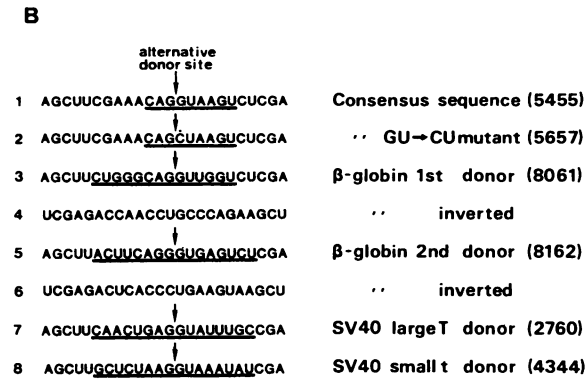

FIG. 1. (A) Structure of the β-globin pre-mRNA substrate carrying an insert as an alternative donor site and possible alternate splicing pathways. In the schematic representation of RNA species, open boxes represent exons, solid boxes represent alternative donor site sequences which have been inserted, and lines represent intron sequences. Partial or total RNA sizes are indicated in nucleotides. Dots indicate RNA species containing a lariat structure with an electrophoretic mobility different from that of a corresponding linear RNA. The mobility on 5% polyacrylamide–7 M urea gel relative to a DNA marker is indicated in parentheses (see Fig. 2). (B) Sequences inserted as alternative donor sites within the intron. Donor sites are indicated by arrows. A consensus 9-nt donor sequence (22) or a donor site sequence from each gene (17, 28) is underlined. All the sequences except 4 and 6 include common sequences AGCUU(5′) and CGA(3′), which were parts of *Hin*dIII and *Acc*I recognition sequences for cloning the original synthetic DNA (37). Sequences 4 and 6 are inverted antisense versions of sequences 3 and 5, respectively. The remaining sequences in 1 and 2 (CGAAA and CU) were arbitrary ones lacking GU, AG, or complementarity to the 5′ end of U1 RNA (37). All the sequences were inserted via an 8-bp *Xba*I linker (CT/CTAGAG) into the *Xba*I site (see Materials and Methods). The numbers in parentheses at the right are those of the original synthetic DNAs (37).

and cleaved with *Xba*I. *Xba*I DNA fragments (32 bp) carrying a short donor sequence (see Fig. 1B) were electrophoretically isolated and cloned into *Xba*I-cleaved, dephosphorylated pSP64-HβUX2. The obtained plasmid clones were verified by the dideoxy sequencing method directly on the double-stranded plasmid with an appropriate primer as described previously (11).

RNA transcription and in vitro splicing. The plasmids were linearized by *Bam*HI and used as the template for in vitro transcription by using SP6 RNA polymerase with a cap primer (12). A standard reaction (25 μl) contained 2.5 μg of DNA template, 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 25 U of placental RNase inhibitor (RNasin, Takara Shuzo), 0.48 mM of m^7GpppG (Pharmacia), 0.5 mM each of ATP and CTP, 50 μM each of GTP and UTP, 25 μCi of [α-^{32}P]UTP (410 Ci/mmol, Amersham Corp.), and 10 U of SP6 RNA polymerase (Boehringer Mannheim Biochemicals). After incubation at 37°C for 2 to 3 h, the RNA product was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and precipitated with ethanol.

A HeLa cell nuclear extract was prepared as described previously (7), with minor modifications. Unless otherwise indicated, the in vitro standard splicing reaction (50 μl) was carried out as described previously (13) except for the reaction time (4.5 h), the volume of a nuclear extract (20 μl), and the deletion of RNasin. The radioactivity of the pre-mRNA added was equivalent to that of 20 fmol of full-size transcript (ca. 1.1×10^5 cpm just after transcription with fresh [^{32}P]UTP). The actual amount of the full-size transcript added was estimated by electrophoresis of the transcripts to be 5 to 7 fmol. After incubation at 30°C, the RNA was extracted by phenol and precipitated with ethanol. The RNA was analyzed on 5% polyacrylamide (acrylamide-bisacrylamide, 19:1)–7 M urea gel followed by autoradiography with an intensifying screen at −80°C. For quantitation of the splicing products, an individual band was cut out from the dried gel and the radioactivity was measured in a liquid scintillation counter. The amount of each RNA was estimated from the specific radioactivity of the RNA molecule and the observed radioactivity from which a background count had been subtracted.

Primer extension analysis. For the preparation of each RNA product, the in vitro splicing reaction was scaled up 48-fold (12 reactions each containing 200 μl). After autoradiography, the RNA was eluted overnight in 0.5 M ammonium acetate–10 mM magnesium acetate–1 mM EDTA–0.1% (wt/vol) sodium dodecyl sulfate with shaking at room temperature and then precipitated with ethanol. Primer extension analysis was done by a modified method of Krainer et al. (13) with one of the following synthetic 20-nt DNAs: primer 1 (CTTGTAACCTTGATACCAAC, complementary to the 5′ end of the intron), primer 2 (CAGTGCCTATCAGAAACCCA, complementary to just upstream of the branch point), primer 3 (CTAAGGGTGGGAAAATAGAC, complementary to the 3′ end of the intron), and primer 4 (AAAGGCACCGAGCACTTTCT, complementary to an internal region of exon 2) (see Fig. 3). Hybridization was done at 45°C overnight in 50 μl containing ca. 0.25 fmol each of isolated RNA product, ca. 0.5 pmol of 5′-end-labeled primer (4×10^5 to 14×10^5 cpm), 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)-NaOH (pH 6.4), 0.4 M NaCl, 1 mM

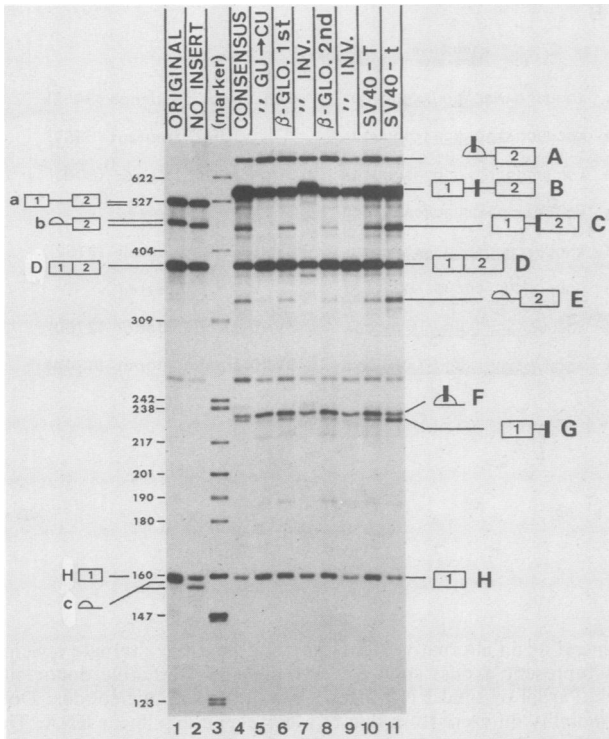

FIG. 2. In vitro splicing of RNA precursors carrying various alternative donor sites under the standard conditions (60 mM KCl–3.2 mM $MgCl_2$). RNAs in the splicing reactions were loaded on 5% polyacrylamide–7 M urea gel and detected by autoradiography. The structures of the RNAs are schematically represented on both sides (see Fig. 1A for symbols). Lane 3, ^{32}P-labeled markers of *Hap*II-digested pBR322 DNA; sizes (in nucleotides) are indicated on the left. Lane 1, Precursor transcribed from the original plasmid pSP64-HβΔ6/*Bam*HI (13). Lane 2, Precursor transcribed from pSP64-HβUX2/*Bam*HI that has an *Xba*I linker but no insert in the *Xba*I site. The precursors in lanes 4 to 11 carry various donor-related sequences (shown in Fig. 1B) within the intron. See Results for identification of the RNAs in the bands. An intron lariat (I) excised at the alternative donor site was not shown in the figure since it ran off the gel (see Fig. 5A). In lanes 1, 2, and 4 to 11, bands above F (corresponding to ca. 260 nt of DNA marker) are seen. They are presumed to contain an RNA consisting of the second exon and a part of the first intron (32).

EDTA, 0.2% (wt/vol) sodium dodecyl sulfate, and 20% (vol/vol) formamide. The hybrids were precipitated with ethanol and dissolved in 20 μl of a reaction mixture containing 50 mM Tris hydrochloride (pH 8.3), 50 mM NaCl, 8 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM each of deoxynucleoside triphosphates, 20 U of RNasin, and 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.). The reaction was performed at 41°C for 1 h, and then the RNA moiety was hydrolyzed in 0.2 N NaOH at 65°C for 1 h. After being neutralized, the elongated DNA was precipitated with ethanol and analyzed on an 8% polyacrylamide–7 M urea gel, followed by autoradiography.

RNA-binding assay. The binding activity for an RNA transcript carrying a donor-related sequence, introduced as an alternative donor site, was assayed by using a filter-binding method as described previously (20, 37) except that the binding reaction was done under the conditions used for splicing. The reaction mixture in 100 μl containing 13 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 0.5 mM ATP, 20 mM creatine phosphate, 0.3 mM dithiothreitol, 130 μM EDTA, 60 mM KCl, 3.2 mM $MgCl_2$, 13% (vol/vol) glycerol, the indicated amount of a nuclear extract (see Fig. 7), and 10 fmol equivalent of ^{32}P-labeled RNA (37 nt) was incubated at 30°C for 20 min and then filtered.

RESULTS

Splicing of RNA precursors including various alternative donor sites. To elucidate the sequence requirement for donor site selection, we prepared human β-globin pre-mRNA derivatives based on the transcript carrying the first exon, the first intron, and a major part of the second exon (shown at the top of Fig. 1A). We introduced the sequences shown in Fig. 1B, which carried possible donor site sequences of 9 to 16 nt, within the first intron (67 nt downstream from the authentic donor site). The pre-mRNAs were tested for the possibility of donor site utilization by an in vitro splicing system with a HeLa cell nuclear extract. First, we spliced all the transcripts in the standard splicing reaction, including 60 mM KCl and 3.2 mM $MgCl_2$, as described previously (13); the results are shown in Fig. 2. From all the precursors carrying an insert (RNA B in Fig. 2, lanes 4 to 11), authentic splicing products (A, D, F, and H) were generated. The structures of RNAs D and H were identified, as shown, since the same RNA species were generated from original and no-insert precursors (Fig. 2, lanes 1 and 2). The structures of A and F were tentatively identified by electrophoretic mobilities, as shown, and confirmed later (see below). Three other RNA species, C, E, and G, were generated from precursors carrying an alternative donor site sequence of consensus, β-globin first or second donor, or SV40 large T antigen (SV40-T) or small t antigen (SV40-t) donor (Fig. 2, lanes 4, 6, 8, 10, and 11). These RNA species were not generated from the precursors carrying the GU→CU mutant donor sequence or an inverted antisense sequence of β-

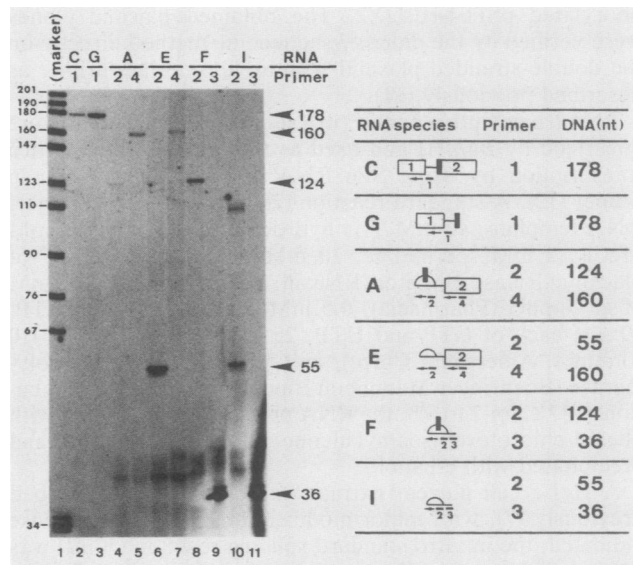

FIG. 3. Primer extension analysis of the splicing products. Isolated splicing products from the precursor carrying the SV40-t donor insert were subjected to primer extension analysis as described in Materials and Methods. The results are summarized schematically on the right (see Fig. 1A for symbols). ^{32}P-labeled markers of pBR322/*Hap*II DNA are shown in lane 1; sizes (in nucleotides) are indicated to the left of lane 1.

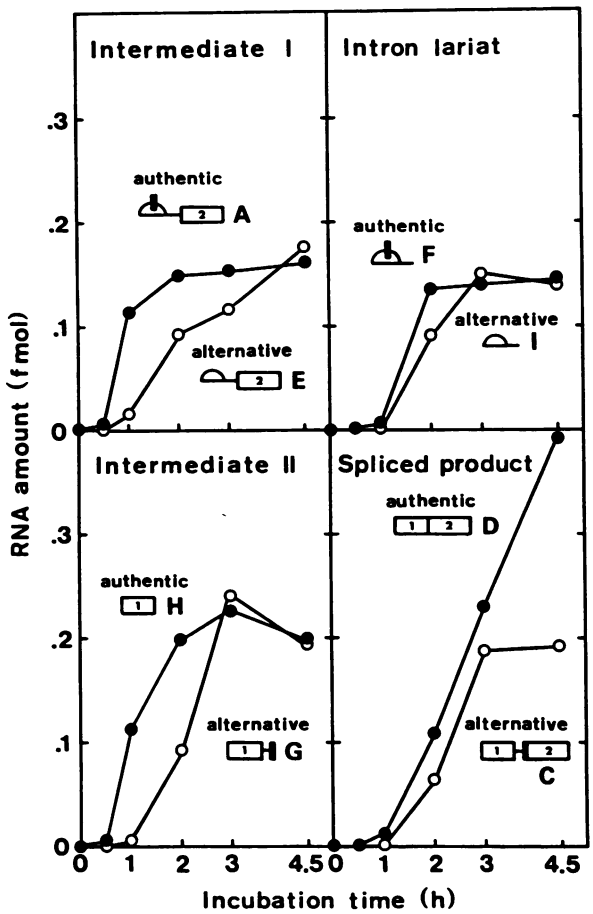

FIG. 4. Time course of in vitro splicing. The precursor carrying the SV40-t donor insert was spliced under the standard conditions (60 mM KCl–3.2 mM $MgCl_2$) for the indicated time. The products were analyzed by electrophoresis on a 5% polyacrylamide–7 M urea gel followed by autoradiography. The amount of each product was estimated as described in Materials and Methods. Letters indicate RNA species; see Fig. 1A for symbols.

globin first or second donor (lanes 5, 7, and 9). The RNA species C, E, and G were presumed to be generated by the alternative use of the insert as the donor site. This prediction was verified by determination of their structures (see next section). Figure 2 shows that the activity of the presumed alternative site depends on its own sequence. Each RNA species was quantitated, and the ratios of the spliced alternative product (C) to the spliced authentic product (D) were calculated for each alternative donor and ranked as follows: SV40-t (C/D, 0.93) > consensus (C/D, 0.51) > SV40-T (C/D, 0.20) > β-globin first (C/D, 0.11) > β-globin second (C/D, 0.03). Similar results were obtained in two other experiments, one with the same nuclear extract and the other with a different preparation. The order of the relative utilization of the alternative site was identical.

Characterization of the splicing products. We analyzed the structures of all the RNA species, which were derived from the pre-mRNA carrying the SV40-t donor and tentatively identified in Fig. 2, by primer extension (Fig. 3). Approximately 178-nt DNA products elongated from primer 1 were observed in both RNA species C and G. These results confirmed the identification of species C as the alternatively spliced product and species G as the first exon plus a part of the intron which was cleaved at the alternative donor site. As for the RNAs E and I, the discrete 55-nt DNA fragments elongated from primer 2 were observed with both species. An approximately 160-nt product of E from primer 4 and an approximately 36-nt product of I from primer 3 were also observed. On the basis of these results and the electrophoretic mobilities, we identified species E as a lariat intermediate cleaved at the alternative donor site and species I as an excised intron lariat derived from E. In the same manner, species A was identified as a lariat intermediate cleaved at the authentic donor site and species F was identified as an intron lariat derived from A. We observed approximately 160-nt products of species A and E from primer 4 and the same products (probably 36 nt) of F and I from primer 3. These results localized the branch point for the alternative splicing and that for the authentic splicing to the same, or nearly the same, position. For the products derived from the pre-mRNAs carrying alternative donors other than SV40-t, their structures were presumed to be the same as those of the corresponding products from the SV40-t pre-mRNA since their electrophoretic mobilities were the same (Fig. 2; see Fig. 5).

Time course of splicing. By using the precursor carrying the SV40-t donor insert that produced the alternatively spliced product most efficiently, the time course of splicing was analyzed (Fig. 4). The intermediates E and G derived from the alternative site accumulated more slowly than A and H derived from the authentic site. As for the spliced products D and C, the rates of accumulation were similar until 3 h. Thus, the rate-limiting step of the alternative product generation appears to be the first step of splicing.

Splicing of RNA precursors at various salt concentrations. To search for factors that affect the activity of the alternative donor site, splicing was examined at 40 and 80 mM KCl for the five pre-mRNAs whose alternative donor sites were used at 60 mM KCl (Fig. 5). With these precursors, the amounts of most of the alternative products were higher at 80 mM KCl than at 40 mM KCl, whereas most of the authentic products were lower at 80 mM than at 40 mM. The ratio of the alternative product to the authentic product was always higher at 80 mM than at 40 mM KCl (Fig. 5B). For the spliced product, the C/D ratio was always higher at a higher KCl concentration in the range from 40 mM to 80 mM (Fig. 2 and 5B). The C/D ratio for the precursor carrying the SV40-t donor insert at 80 mM was much higher than 1 (2.0). Splicing of the precursor carrying the consensus donor insert was analyzed under conditions where the concentrations of both $MgCl_2$ (1.6 to 4.8 mM) and KCl (40 to 100 mM) were varied (Fig. 6). Effective splicing was observed at 100 mM KCl, in contrast to the previous reports from other laboratories (13, 34). Figure 6 also shows that at all the $MgCl_2$ concentrations tested, the ratio of alternative product to corresponding authentic product increased according to the KCl concentration. Under several high-salt conditions, the C/D ratio was higher than 1. A similar dependence on salt concentration was observed with the precursor carrying the SV40-t donor insert under conditions of 40, 60, and 80 mM KCl and 1.6, 3.2, and 4.8 mM $MgCl_2$ (data not shown). Under several high-salt conditions, the C/D ratio was higher than 1. At 6.4 mM $MgCl_2$, both authentic and alternative splicing were significantly suppressed.

Binding activity to the alternative donor site sequence. To obtain an index of donor site strength, we assayed the binding activity of the splicing extract for the donor site sequence inserted at the alternative site (see Fig. 1B) by the method described previously (37). Figure 7 shows the binding to the short RNAs carrying the donor site sequences

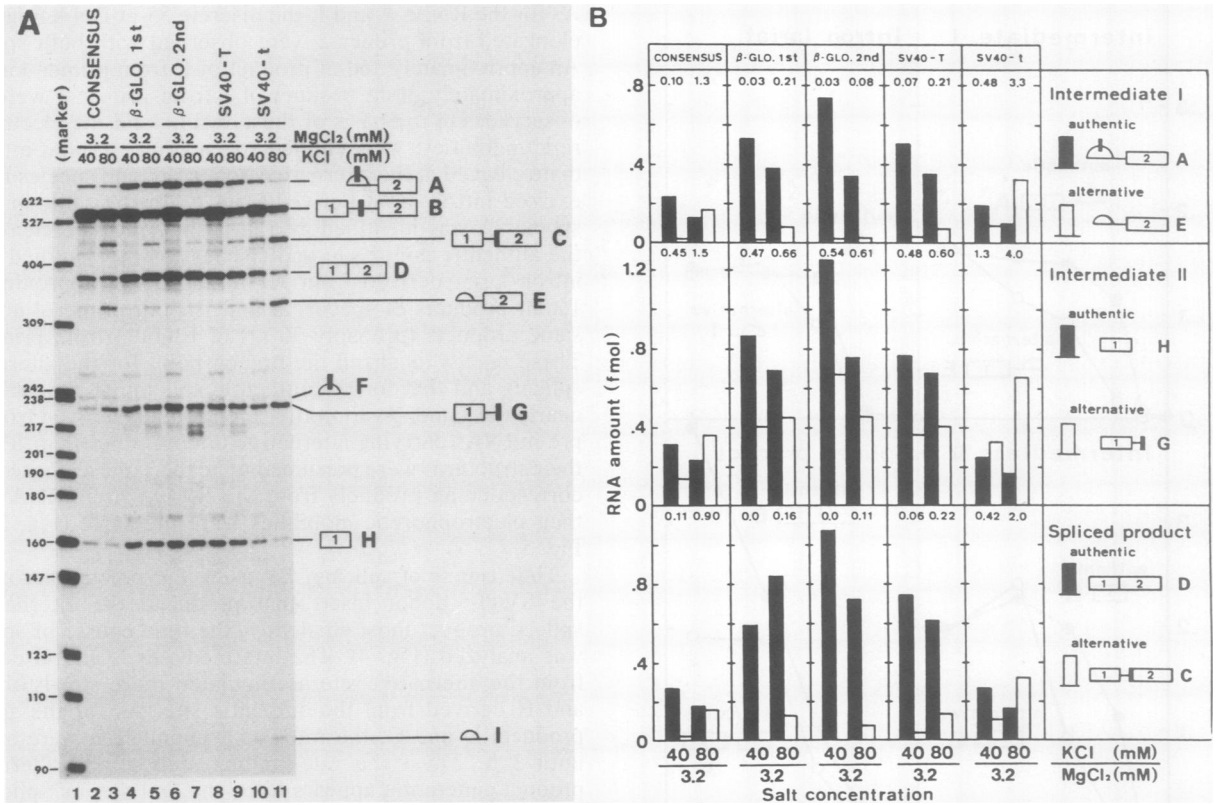

FIG. 5. (A) In vitro splicing of RNA precursors carrying various alternative donor sites at low (40 mM) and high (80 mM) KCl concentrations. The structures of the RNAs are schematically represented on the right (see Fig. 1A for symbols). (B) The amount of each RNA product shown in panel A was quantitatively estimated as described in Materials and Methods. The ratio of the alternative product to the authentic product is shown at the top of each column. The corresponding RNA species is shown on the right. See Fig. 1A for symbols.

under the standard conditions for splicing. These results are consistent with our previous data obtained with a partially purified U1-rich small nuclear ribonucleoprotein particle fraction (37). The free energy increments (ΔG) calculated for the base pairing between the 5′-end region of U1 RNA and the donor site sequences are −18.3 kcal for consensus, −13.8 kcal for SV40-t, −11.7 kcal for β-globin second, −11.1 kcal for β-globin first, −10.8 kcal for SV40-T, and −10.5 kcal for the GU→CU mutant (37). The relative utilization of an alternative donor site (Fig. 2) is not necessarily in parallel with the binding activity shown in Fig. 7, nor is it in parallel with the free energy increment for the base pairing stated above.

DISCUSSION

When we discuss the mechanism of splice site selection, there are many factors to be considered, even though these are limited to *cis*-acting factors. The intrinsic strength of a splice site (8, 40), its position (15, 16, 23, 27, 30), the length of an intron (9, 33, 39), adjacent exon sequences (30), and the secondary structure of a pre-mRNA (4, 31, 35, 36) have been proposed as important determinants of splice site selection. To gain knowledge about the factor required for splice site selection, we carried out *cis* competition assays between two donor splice sites in vitro. For purposes of simplification, we systematically varied a single factor while maintaining the other factors constant. We introduced one of several donor site fragments into a constant position within the intron of a β-globin transcript and examined the utilization of these fragments for splicing in vitro.

A short donor sequence can serve as an active donor site signal. Each of the five possible donor site sequences inserted was variably active as an alternative donor site, at least in splicing with 60 mM–3.2 mM $MgCl_2$ and 80 mM KCl–3.2 mM $MgCl_2$ (Fig. 2 and 5). Under certain conditions used, consensus and SV40-t donor inserts served for splicing more efficiently than did the authentic β-globin donor sites. The same branch point was probably used for both splicings (Fig. 3). These results indicate that a relatively short sequence inserted within the intron can actually serve as a donor in vitro, even in the presence of the authentic β-globin donor site. A sequence tested by insertion within the intron consists of 24 nt (Fig. 1B) flanked by 8 nt of *Xba*I linker. The inserted sequence, however, contains only 9 to 16 nt (underlined in Fig. 1B) which were derived from sequences around a donor site. These consist of 3 or 8 nt of an exon and 6 or 8 nt of an intron. Therefore, the results we obtained suggest that the 9-nt consensus sequence and 16- or 14-nt sequences from β-globin and SV40 donors contain a signal which is adequate as a donor site in splicing. Our results are in line with the following reports. (i) Duplicated short large T antigen and small t antigen donor site sequences in SV40 transcripts, although they contain only 21 and 11 nt of adjacent exon sequences, respectively, were the preferred splice site in vivo (9). (ii) No more than approximately 20 nt of either exon was necessary for efficient splicing in vitro in the rabbit β-globin transcript (26). (iii) The 9-nt consensus donor site sequence inserted within the exon upstream of the second donor site in rabbit β-globin transcript was used efficiently in vivo (8). In this last study, however, the

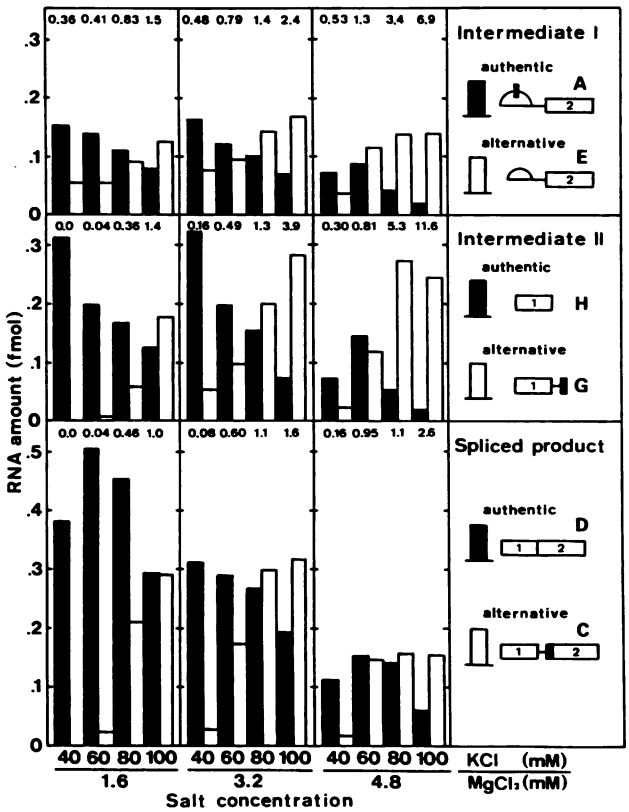

FIG. 6. Effect of KCl and $MgCl_2$ concentrations on donor site utilization during splicing in vitro. Splicing products from the precursor carrying a consensus donor insert under various salt conditions were quantitatively analyzed as described in Materials and Methods. The ratio of the alternative product to the authentic product is shown at the top of each column. The corresponding RNA species (denoted by letters) are shown on the right. See Fig. 1A for symbols.

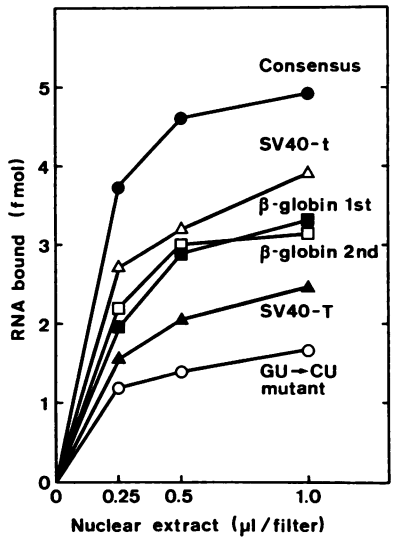

FIG. 7. Binding activity of a nuclear extract to the 37-nt RNAs carrying the single donor-related sequence shown in Fig. 1B. The binding reaction was carried out under the standard splicing conditions (60 mM KCl–3.2 mM $MgCl_2$) with the indicated amount of nuclear extract at 30°C for 20 min.

efficiencies of the test (alternative) donors relative to those of the reference (authentic) donor sites exceeded those in our results. This discrepancy may be partly due to the presence of an exon sequence adjacent to their inserts (see below), the difference in the intrinsic efficiency of the reference sites, the difference in the distance between the test site and the reference site (25 nt versus 67 nt in our case), or the difference between in vivo and in vitro conditions.

Our results concerning the relative use of β-globin first donor insert is consistent with those of Reed and Maniatis (30), although the implications of our overall results for the relative contribution of exon sequences may be different from theirs. More recently, Nelson and Green (23) reported that consensus and rabbit β-globin donor site sequences of 9 nt, which were inserted in the second exon or intron in a rabbit β-globin transcript, were inactive in vitro in three positions out of four if the authentic donor site was present. In the absence of the authentic donor site, the consensus donor insert was active in all the positions, and the activity of rabbit β-globin donor insert was position dependent. Therefore, they proposed that the 9-nt donor site sequence is not sufficient for splice site utilization and that the region outside of the 9 nt, or "splice site context," is important. The apparent discrepancy between our results under the same standard conditions (60 mM KCl–3.2 mM $MgCl_2$) and those of Nelson and Green may be due to a difference in the regions outside of the 9-nt inserted donors. If these regions in our precursor, which were derived either from the extra sequence within the insert or from the sequences surrounding the insertion site within the intron, carry the "donor site context," our results could be reconciled with those of Nelson and Green. These possibilities should be tested. On the other hand, it seems possible to us that their inserted sequences could be used under different salt conditions even in the presence of the authentic donor site.

Efficiency of inserted donor site depends on its own sequence. As already indicated by other investigators (8, 9, 23, 40), the activity of an inserted sequence as a donor site depended on its own sequence (Fig. 2 and 5). The splicing at 60 mM KCl–3.2 mM $MgCl_2$ (the standard condition) and at 80 mM KCl–3.2 mM $MgCl_2$ ranked the activities of the donor site sequences tested as follows: SV40-t > consensus > SV40-T > β-globin first > β-globin second. This hierarchy was not in parallel with that of the binding activity of the donor sequences to the splicing extract measured under the standard conditions (Fig. 7). Therefore, we suggest that the intrinsic efficiency of a donor site is not simply dependent on its binding to a donor site factor, or U1 small nuclear ribonucleoprotein (37), but is also dependent on the efficiency of later steps, such as interaction with other factors, as described by Chabot and Steitz (5), and cleavage-ligation reactions. This result is supported by Nelson and Green (23), who showed that the inserted splice sites were bound by the appropriate splicing factors (U1 small nuclear ribonucleoprotein or 70-kilodalton RNA-binding protein) regardless of its utilization in splicing.

Efficiency of inserted donor site depends strongly on the concentrations of both KCl and $MgCl_2$. In most of the results shown in Fig. 5 and 6, the authentic spliced product decreased and the alternatively spliced product increased at higher concentrations of KCl. This inverse relation between the two products suggests competition for splicing between the two donor sites. A possible explanation may be the competition between the two donor site-factor complexes to interact with the single acceptor site-factor complex, as

proposed by Eperon et al. (8). The C/D ratio increased when the KCl concentration increased within the range tested (Fig. 5 and 6; also see Results). A similar dependence of the C/D ratio on the $MgCl_2$ concentration was observed in the range from 1.6 to 4.8 mM at a fixed KCl concentration (Fig. 6 and data not shown). At 6.4 mM $MgCl_2$, both the authentic and alternative splicings were greatly reduced (by the precursor carrying SV40-t donor insert; data not shown). The possibility of a reduced specificity of donor site recognition under high-salt conditions is unlikely, because the two cryptic donor sites found in the β-globin first exon (38) were not activated under high-salt conditions. Our results are consistent with those of Schmitt et al. (34) in the sense that a proximate donor site is more preferred at a higher salt concentration.

Possible mechanisms for the more efficient utilization of alternative donors at higher KCl concentrations are as follows. (i) Stabilization of base pairing between these donor sites and U1 RNA (2, 14, 37, 41) may be due to the salt. The calculated intensity of this base pairing with the consensus donor is the highest and that with the SV40-t is the second highest among the five donors tested here (37). It is likely that the effect of salt in stabilizing the base pairing may be more pronounced for a stronger base pairing. While the base-pairing stabilization by the salt can explain the highly efficient utilization of consensus and SV40-t alternative donors at higher KCl concentrations, it cannot explain the more efficient utilization of the β-globin first donor insert at a higher salt concentration, which should have the same stabilizing effect as the authentic donor site. Presumably another mechanism common to all five alternative donors is required. (ii) A secondary structure of the precursor, which depends on the specific position of the alternative donor site and is favorable for the interaction between the alternative site and the acceptor, is preferred at a higher KCl concentration. If this were the case, even if the alternative site were more distant from the acceptor than the authentic site, such a dependence on salt as shown here could be observed if a relevant secondary structure were involved. (iii) An interpretation of our results is that a donor site proximate to an acceptor is increasingly favored at a higher salt concentration. If this were the case, a plausible explanation may be that a salt-sensitive and intron-size dependent reaction, e.g., protein-RNA interaction for intron scanning, is involved in the splicing.

Possible biological significance of salt concentrations. Both authentic and alternative splicings were observed at 80 mM and 100 mM KCl with all the precursors tested (Fig. 5 and 6). In vitro splicing of the original human β-globin transcript (Fig. 2, lane 1) was not detected at 100 mM KCl (13), and KCl concentrations higher than 60 mM impaired in vitro splicing of an adenoviral transcript (34). The apparent discrepancy between our results and those of other investigators may be due either to differences in the splicing extracts used or to the precursors or both. However, it seems reasonable that splicing can occur efficiently at 80 mM and 100 mM KCl, since the physiological K^+ concentration is higher than 100 mM (1). The total cellular magnesium concentration is variably reported (19) as is the intracellular free Mg^{2+} concentration (1, 6). Since the physiological Mg^{2+} concentration seems to vary depending on the cell type and on the extracellular medium (1), Mg^{2+} may be a better candidate than K^+ as a regulatory factor for alternative splicing.

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