β-globin transcripts carrying a single intron with three adjacent nucleotides of 5′ exon are efficiently spliced *in vitro* irrespective of intron position or surrounding exon sequences

Akila Mayeda+ and Yasumi Ohshima*
Department of Biology, Faculty of Science, Kyushu University, Hakozaki, Higashi-ku, Fukuoka 812, Japan

Received June 26, 1990; Accepted July 17, 1990

ABSTRACT

To examine the role of exon sequences and intron position in the splicing of an mRNA precursor, we prepared series of sense or anti-sense transcripts of human β-globin cDNA in which a cassette containing the β-globin first intron was inserted into one of seven unusual positions. The intron cassette consisted of the intron alone (mI), the intron with three adjacent base pairs of the 5′ exon (MI), or the intron with both 5′ and 3′ exon sequences. All these transcripts were examined in an *in vitro* splicing system with a HeLa cell nuclear extract. The sense transcripts carrying MI cassette were spliced efficiently and independently of the intron position, except when the 3′ exon was too short. The anti-sense transcripts carrying MI cassette produced significantly less spliced products than did those of the sense transcripts. This was mostly because of the instability of the anti-sense transcripts, and the actual splicing efficiency was similar to that seen in the sense transcripts. Sense or anti-sense transcripts carrying mI cassette were spliced to various extents depending on the surrounding sequences. The results indicate that only three nucleotides of the 5′ exon are required as specific exon sequences in the splicing of an mRNA precursor carrying a single intron, and that the intron position does not significantly affect the splicing efficiency *in vitro*.

INTRODUCTION

Sequences around the 5′ and 3′ splice sites and those around the branch point of an mRNA precursor (pre-mRNA) carry signals that are important to determine the site and efficiency of the splicing (1−3). These signals are known to reside mostly within an intron: 3 nucleotides (nt) of the 5′ exon and 1 nt of the 3′ exon are conserved (1−5). Are there any other generally important signals in a mammalian exon? The role of a long adjacent exon sequence (several tens of nucleotides) in the selection of one of the two identical splice sites was shown *in vitro* (6). The 'sequence context' required for efficient utilization *in vitro* of an inserted splice site sequence (7) could, at least partly, reside within an exon sequence. On the other hand, exon truncation studies limited the minimum size of the exons required for efficient splicing to 21 or 24 nt for the 5′ exon (8, 9) and 21 nt (9) or more than 24 nt (10) for the 3′ exon. Exon replacement experiments *in vitro* done by Parent *et al.* (9) suggested that the upper limits of the specifically required sequences in these exons were 10 nt and 1 nt, respectively. Other insertion experiments *in vivo* (11) or *in vitro* (12) showed that 9 nt sequences were sufficient as a 5′ splice site signal at least in some cases. Thus, the precise requirement for exon signals in mammalian pre-mRNA splicing has not been determined. As a new approach to elucidate this problem, we constructed several series of sense or anti-sense transcripts of human β-globin cDNA in which an intron cassette was inserted at one of various positions. The intron cassette carries the first intron with or without exon sequences of the human β-globin gene. The splicing of all these transcripts was examined *in vitro*. These experiments also served to clarify the effect of intron position within pre-mRNAs of the same size, which is another subject of interest. A report of this kind, in which splicing was examined in yeast *S. cerevisiae* (13), was published. However, no mammalian or *in vitro* studies have been reported.

MATERIALS AND METHODS

Construction of intron cassettes

The intron cassettes consisting of the 130 base pairs (bp) human β-globin first intron alone (mI) or the first intron plus the adjacent 3 bp of the 5′ exon (MI, 133 bp) were constructed from nine synthetic oligonucleotides. Each oligonucleotide (17−49 mer) was chemically synthesized by an automated DNA synthesizer (Applied Biosystems model 380B) and purified by high

* To whom correspondence should be addressed

+ Present address: Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, NY 11724, USA

performance liquid chromatography (Applied Biosystems model 152A). After phosphorylation by T4 polynucleotide kinase (Takara Shuzo), seven oligonucleotide components for mI or MI (100 pmol each) were annealed at 70°C for 5 min in the presence of 100 mM Tris-HCl (pH 7.5)/1 M NaCl followed by slow cooling to room temperature. The annealed products were diluted 100-fold with 100 mM Tris-HCl (pH 7.5)/5 mM $MgCl_2$ for blunt-end ligation. The intron cassettes with the adjacent 5′/3′ exon sequences of approximately 8/12 bp (daI) and 89/116 bp (DAI) were excised from pSP64-HβΔ6(ΔBamHI) plasmid by *Eco*O109/*Acc*I and *Nco*I/*Ban*I cleavage, respectively (Fig. 1).

Construction of plasmids

The scheme for construction of HβE series plasmids for sense transcripts (7×4=28 kinds) is illustrated in Fig. 1. The pSP65-HβF plasmid vector for anti-sense transcripts (Fig. 5) was made by recloning the *Hin*dIII-*Eco*RI fragment of pSP64-HβE (Fig. 1) between the *Eco*RI and *Hin*dIII sites of pSP65 (14). Plasmids of pSP65-HβF series in which the intron cassette (mI or MI) was inserted into one of seven positions were also constructed (7×2=14 kinds). All the candidate clones were verified by the dideoxy sequencing method with an appropriate primer, as described (15).

RNA transcription and *in vitro* splicing

The HβE and HβF series plasmids were linearized by *Eco*RI and *Hin*dIII, respectively, and used as the templates for *in vitro* transcription with SP6 RNA polymerase (Takara Shuzo) and GpppG cap (Pharmacia) as a primer (12).

A nuclear extract from HeLa cells was prepared as described (16) with minor modifications. The *in vitro* splicing reaction was carried out at 30°C for 2 hours (h) with 20 fmol of ^{32}P-labeled transcript in 25 μl as described (17), except for the volume of nuclear extract (10 μl), the absence of RNasin (placental RNase inhibitor), and addition of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES)-KOH (pH 7.3). This pH adjustment significantly increased the splicing activity (unpublished data). The splicing products were analyzed in a denaturing 5% polyacrylamide gel followed by autoradiography. The results of *in vitro* splicing presented here were obtained with a single nuclear extract preparation. The results were also confirmed in other experiments using two other preparations of nuclear extract, with close agreement. The splicing of transcripts derived from β-globin gene has already been well characterized (17−20). Therefore, splicing products in our experiments were identified by their electrophoretic mobility and comparison with those from the wild type (Wt) transcript.

RESULTS

Effect of adjacent exon sequences on splicing efficiency

We first attempted to prepare four series of sense transcripts of human β-globin cDNA in which daI, DaI, dAI, or DAI intron cassette (β-globin first intron plus approximately 8/12 bp, 89/12 bp, 8/116 bp, or 89/116 bp of the adjacent exons) was inserted at each of seven unusual positions (see Fig. 1). Most of these templates were constructed, and their transcripts were tested in an *in vitro* splicing system with a HeLa cell nuclear extract. All of them were efficiently spliced as was the Wt transcript carrying the first intron at the inherent position (data not shown), indicating that 8 and 12 nt of the 5′ and 3′ exon, respectively, were sufficient as specific exon signals. Therefore, we also prepared transcripts

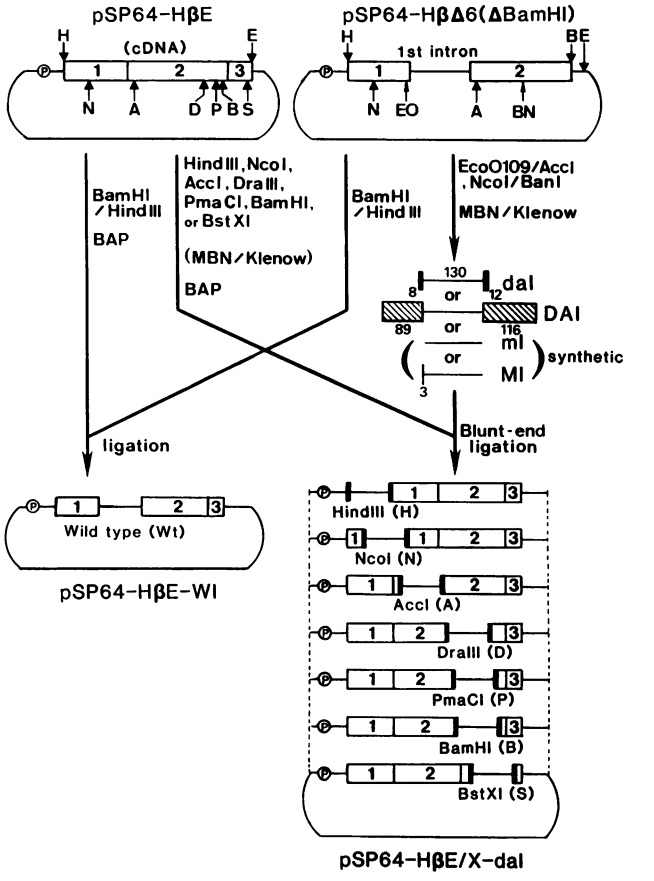

Figure 1. Schematic representation of the construction of template plasmids for pre-mRNA trancription. The plasmids include β-globin cDNA in which one of the intron cassette is inserted at each of seven different positions. Here we show only one of the constructed HβE plasmids including dal cassette (pSP64-HβE/X-dal) as an example. pSP64-HβE was derived from the cDNA plasmid pSP64-HβD6-IVS1,2 (17) with deletion of an *Eco*RI fragment containing the 3′ non-coding region. pSP64-HβΔ6(ΔBamHI) was derived from pSP64-HβΔ6 (17) by recloning the *Hin*dIII-*Bam*HI fragment into pSP64 (14). Except for *Eco*O109 (EO) and *Ban*I (BN), all the other restriction sites indicated are unique. The transcript from pSP64-HβE-WI was used as a control and termed 'wild type (Wt)' here, despite the lack of the second intron. MBN; mung bean nuclease. Klenow; Klenow fragment of *E. coli* DNA polymerase I. BAP; bacterial (*E. coli*) alkaline phosphatase.

carrying mI (intron alone) or MI (intron plus 3 bp of the 5′ exon) cassette and examined the splicing. Splicing results for transcripts carrying mI, MI, daI, or DAI cassette at two different positions are shown as typical examples (Fig. 2). Transcripts carrying MI, daI, or DAI cassette were spliced as efficiently as the Wt transcript. There were no significant differences in splicing efficiency among the intron cassettes (MI, daI, and DAI) or positions inserted (N and B sites). In contrast, mI intron without any adjacent exon sequence was spliced out only slightly (N site insertion) or not at all (B site insertion). These results suggested that only 3 nt (CAG) of the specific 5′ exon sequences are required and that the position of an intron has no significant effect on its removal.

Significance of the 3 nt 5′ exon sequence and effect of intron position

To examine the role of exon sequences and intron position in more detail, the MI and mI cassettes were inserted into each of seven different positions of β-globin cDNA (Figs. 1 and 3). All

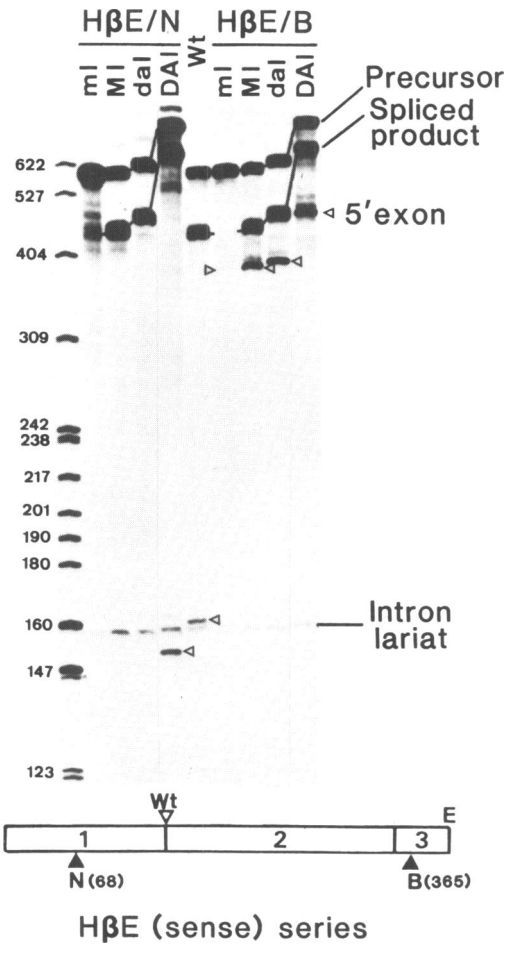

Figure 2. *In vitro* splicing of sense (HβE) transcripts carrying one of four kinds of intron cassette (mI, MI, dal, or DAI) at N or B position. Splicing products were analyzed as described in Materials and Methods. Precursors and mature spliced products are connected by the line. Released 5′ exon (◁) and excised intron lariat are indicated. Size markers of *Hpa*II-digested pBR322 DNA fragments are indicated on the left with their sizes (nt). Positions in the precursor RNA for the insertion of an intron cassette are shown below, with the distances (nt) from the 5′ end in parentheses. See Fig. 1 for the abbreviations.

			5′exon	5′ss	intron	3′ss	3′exon
HβE (sense)	H	mI	GpppGAAUACAAGCU	GU (-4.9)		AG	AGCUUGCUUACAN415
		MI	2NAUACAAGCUCAG	GU (-11.3)		AG	AGCUUGCUUACAN415
	N	mI	56NAACAGACACCAU	GU (-7.5)		AG	CAUGGUGCACCUN357
		MI	60NGACACCAUGCAG	GU (-11.3)		AG	CAUGGUGCACCUN357
	A	mI	160NUGCUGGUGGUCU	GU (-4.9)		AG	CUACCCUUGGACN252
		MI	163NUGGUGGUCUCAG	GU (-11.3)		AG	CUACCCUUGGACN252
	D	mI	319NCCUUUGCCACAC	GU (-7.5)		AG	GUGAGCUGCACUN88
		MI	322NUUGCCACACCAG	GU (-11.3)		AG	GUGAGCUGCACUN88
	P	mI	348NGACAAGCUACAC	GU (-7.5)		AG	GUGGAUCCUGAGN61
		MI	351NAAGCUACACCAG	GU (-11.3)		AG	GUGGAUCCUGAGN61
	B	mI	353NGCUACACGUGGA	GU (-4.9)		AG	GAUCCUGAGAACN59
		MI	358NACGUGGAUCCAG	GU (-11.3)		AG	GAUCCUGAGAACN59
	S	mI	405NGUGCUGGCCCAU	GU (-7.5)		AG	GCAAAGAAUU
		MI	407NGCUGGCCCACAG	GU (-11.3)		AG	GCAAAGAAUU
HβF (anti-sense)	E	mI	2NAUACACGGAAUU	GU (-4.9)		AG	AAUUCUUUGCCAN415
		MI	5NCACGGAAUUCAG	GU (-11.3)		AG	AAUUCUUUGCCAN415
	S	mI	9NGAAUUCUUUGCC	GU (-4.9)		AG	AUGGGCCAGCACN398
		MI	13NUCUUUGCCACAG	GU (-11.3)		AG	AUGGGCCAGCACN398
	B	mI	67NAAGUUCUCAGGA	GU (-4.9)		AG	GAUCCACGUGUAN348
		MI	71NUCUCAGGAUCAG	GU (-11.3)		AG	GAUCCACGUGUAN348
	P	mI	72NCUCAGGAUCCAC	GU (-7.5)		AG	GUGUAGCUUGUCN341
		MI	75NAGGAUCCACCAG	GU (-11.3)		AG	GUGUAGCUUGUCN341
	D	mI	98NAGUGCAGCUCAC	GU (-7.5)		AG	GUGUGGCAAAGGN312
		MI	101NGCAGCUCACCAG	GU (-11.3)		AG	GUGUGGCAAAGGN312
	A	mI	262NGUCCAAGGGUAG	GU (-10.2)		AG	AGACCACCAGCAN153
		MI	265NCAAGGGUAGCAG	GU (-11.3)		AG	AGACCACCAGCAN153
	N	mI	366NCAGGUGCACCAU	GU (-7.5)		AG	CAUGGUGUCUGUN50
		MI	369NGUGCACCAUCAG	GU (-11.3)		AG	CAUGGUGUCUGUN50
HβE	Wt		146NGGCCCUGGGCAG	GU (-11.3)		AG	GCUGCUGGUGGUN264
HβE (no intron)			146NGGCCCUGGGCAG				GCUGCUGGUGGUN264
HβF (no intron)			274NACCACCAGCAGC				CUGCCCAGGGCCN139

Figure 3. Adjacent exon sequences of the sense (HβE) or anti-sense (HβF) transcripts carrying mI or MI intron cassette at one of seven different positions. The intron sequence is identical with that of the β-globin first intron. Adjacent 3 nt (CAG) of 5′ exon are all conserved in MI cassette while they depend on intron position in the transcript carrying mI cassette. The 3′ exons in all the transcripts depend on the intron position. As an index of the 5′ splice site strength, the free energy increments (ΔG) are calculated for the base pairing between the 5′ end region of U1 snRNA (ACΨΨACCUG) and the conserved 9 nt sequences at the 5′ splice site (39). These values in kcal are indicated in parentheses.

these transcripts (HβE series) were tested for splicing (Fig. 4) and the spliced products were quantitated (data not shown). The transcripts carrying MI cassette at six of the seven positions (except S site) were spliced as efficiently as, or more efficiently than, the Wt transcript. Slight differences in the size of the precursors and spliced products observed reflect the size differences generated in the template construction (see Figs. 1 and 3). In the transcript with MI insertion at S site, only the first step of splicing, *i.e.* cleavage at the 5′ splice site and formation of the branched intermediate, occurred. Consequently, the long released 5′ exon was seen slightly below the spliced product. Absence of the mature spliced product was confirmed by the absence of excised intron lariat (Fig. 4, compare with the products from other transcripts). This result is due to the short length of the 3′ exon (10 nt, see Fig. 3) and is consistent with the previous observations that the truncated 3′ exon of 5−14 nt inhibited the second step but not the first step of splicing in the β-globin transcript (9, 10, 19).

On the other hand, transcripts carrying mI cassette were spliced with various degrees of efficiency, depending on the position of insertion. Transcripts with mI insertion at N, D, or P site were efficiently spliced. The transcript with H site insertion was not spliced at all, at least partly because of the insufficient length of the 5′ exon (11 nt). Thus, we propose that the minimum size of 5′ exon is at most 14 nt, which is 7 or 10 nt shorter than that previously reported (8, 9), since the transcript with MI insertion at H site (containing 14 nt of 5′ exon) was efficiently spliced (Fig. 4). As in the case with MI insertion, only the first step of splicing occurred with the S site insertion, which leaves only 10 nt of 3′ exon. The transcript with mI insertion at B site was spliced, but with a very low efficiency (Fig. 4). The transcript with mI insertion at A site, just downstream of inherent first intron position, was not spliced at the 5′ end of the inserted intron, but was spliced *via* an activated cryptic 5′ splice site (Fig. 4, see Discussion). The results presented here show that when the intact intron is flanked by three conserved nucleotides (CAG) of 5′ exon, the intron is always efficiently spliced out, regardless of surrounding exon sequences or position, except when the 5′ or 3′ exon is too short.

Position independent role of the natural exon sequence

Even though the surrounding exon sequences except for 3 nt of the 5′ exon do not significantly affect the splicing efficiency, we cannot rule out the possibility that some or the overall exon sequence might have a role in splicing, which is independent of its position relative to the intron. To examine this possibility, we also inserted the intron cassette, ml or MI, into cDNA which

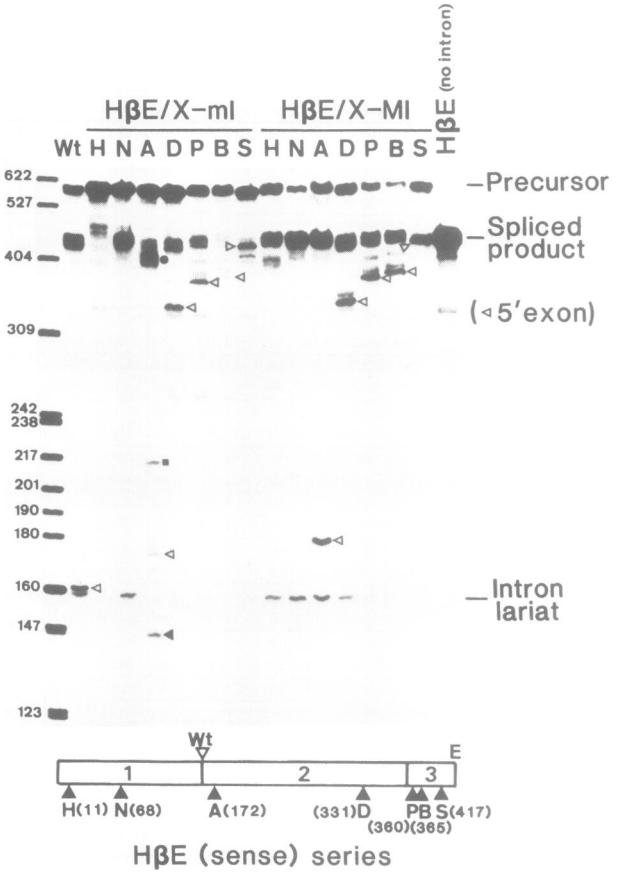

Figure 4. *In vitro* splicing of sense (HβE) transcripts carrying mI or MI intron cassette at various positions. Cryptic spliced product (●) accompanied by the corresponding 5′ exon (◀) and intron lariat (■) are indicated (see text). Positions for intron cassette insertion are shown below with the distances from the 5′ end in parentheses. Quantitation of the splicing products was performed as described (12). See legend to Fig. 2 for other descriptions.

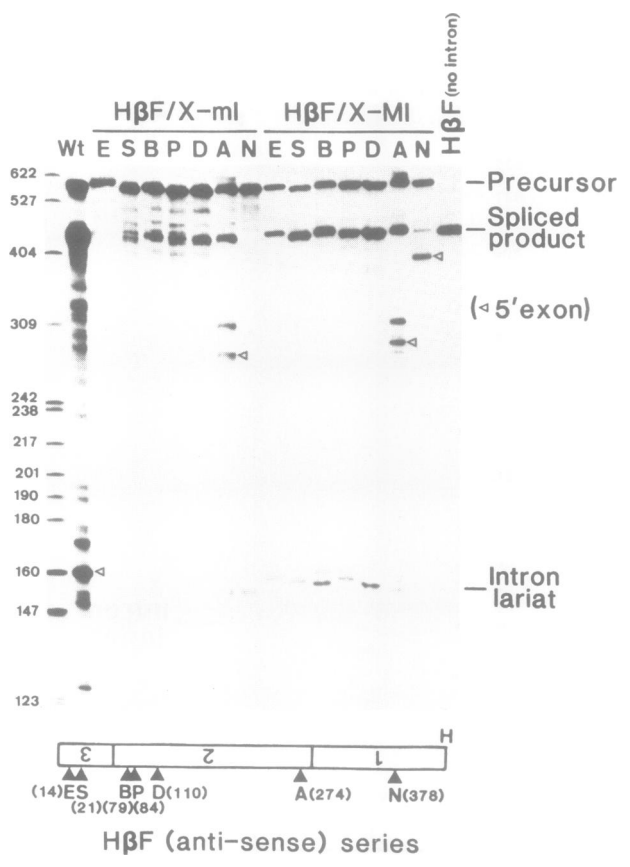

Figure 5. *In vitro* splicing of β-globin anti-sense (HβF) transcripts carrying mI or MI intron cassette at various positions. The positions for intron cassette insertion are shown below with the distance (nt) from the 5′ end in parentheses. In the transcripts with A site insertion, products are seen above the excised 5′ exon, but their nature is unknown. See legends to Fig. 2 for the other descriptions.

had been subcloned in the reverse orientation in the template plasmid. Since the anti-sense (HβF) RNA is considered to include quite different sequence information from that of sense (HβE) RNA, we can examine a position independent role of the exon sequence of the β-globin gene. Remarkably, the transcripts carrying MI cassette at any position were spliced (Fig. 5; see discussion for a lower splicing efficiency of the transcript with N site insertion). Anti-sense transcripts with mI insertion at positions B, P, D, or A were efficiently spliced (Fig. 5). Anti-sense transcripts with mI insertion at positions E, S, or N were not spliced or were spliced inefficiently. The slight differences in size of precursors and spliced products observed reflect the size differences generated in the template construction.

It should be noted that amounts of the spliced products from the anti-sense (HβF) transcripts were significantly lower than those from the sense (HβE) transcripts (see Wt transcript in Figs. 4 and 5 for common control). This phenomenon was probably due to the instability of anti-sense (HβF) RNAs, because survival of the intron-less HβF transcript corresponding to the spliced product (right side lane of Fig. 5, 0.33 fmol) was much less than that of intron-less HβE transcript (right side lane of Fig. 4, 4.6 fmol) under the same splicing condition. The ratio of the amount of the spliced product to that of the survived intron-less transcript was 26–68% for HβE-MI series except for S site insertion (Fig. 4) and 15–97% for HβF-MI series except for N site insertion (Fig. 5). Therefore, no significant differences were found in the splicing efficiency itself between HβE and HβF transcripts, although the accumulated product levels differed greatly. Thus, we can probably rule out the possibility that the overall exon sequence plays a position-independent role in the excision of the intron.

Stability of sense and anti-sense RNA

To further characterize the stability of the β-globin sense (HβE) and anti-sense (HβF) RNA, the intron-less transcripts corresponding to the spliced products were incubated for different times under the splicing reaction conditions (Fig. 6). In contrast to HβE RNA, HβF RNA was rapidly degraded with an increase of incubation time. It is noteworthy that degradation of the HβE RNA was mostly arrested after incubation for 1 h, while progressive degradation continued with HβF RNA.

DISCUSSION

The observations presented here were obtained by *in vitro* splicing of the human β-globin sense or anti-sense transcripts in which a single intron cassette was inserted into various regions. Thus, the proposed conclusions do not always apply to splicing of pre-mRNAs including multiple introns or alternative splice sites.

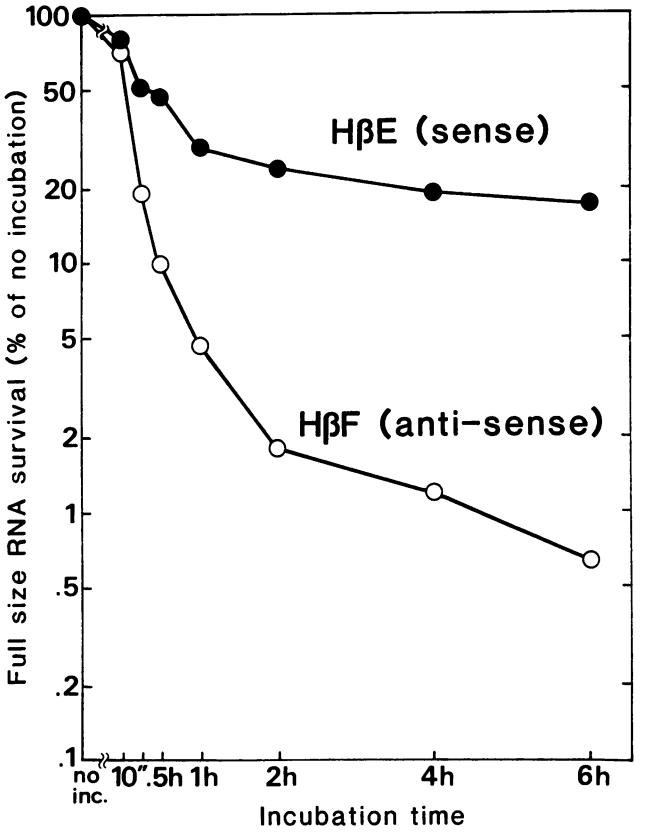

Figure 6. Stability of the sense and anti-sense RNA under the *in vitro* splicing condition. Intron-less sense (HβE) and anti-sense (HβF) RNAs were incubated for the indicated time in the same reaction conditions as in Figs. 4 and 5. The amounts of surviving full size RNA were quantitated as described (12), and presented as a percentage of that seen in the case of no incubation, on a logarithmic scale.

Requirement for specific exon sequence in the splicing of a pre-mRNA

The anti-sense transcript carrying MI cassette at N site was appreciably spliced albeit at a reduced efficiency (Fig. 5, see the later discussion regarding this reduced efficiency). All the other sense or anti-sense transcripts with MI cassette insertion, and with sufficient exon length, were efficiently spliced (Figs. 4 and 5). In contrast, several sense or anti-sense transcripts carrying the intron alone (mI cassette) showed little or no splicing (Figs. 4 and 5). Therefore, it appears that CAG, the 3 nt exon sequence of the authentic 5′ splice site, is necessary and sufficient for splicing as the natural 5′ exon sequence. The efficiently spliced sense or anti-sense transcripts with mI insertion have CAU, CAC, UAG, or GGA as the 5′ adjacent sequence, and those not efficiently spliced have UCU, AUU, GCC, or GGA (Figs. 3–5). Our results suggest that CAU, CAC, and UAG, which have 2/3 match to the consensus CAG, support splicing out of the β-globin first intron (with GUUGGU at the 5′ end), and UCU, AUU, and GCC, which have no match to CAG, do not support. The free energy increments for the presumed base pairing between the 5′ splice site and U1 small nuclear (sn)RNA are −7.5 kcal or lower for the former efficiently spliced transcripts except for the GGA transcript (−4.5 kcal), and −4.5 kcal for all the latter transcripts (Fig. 3). This overall correlation between the intrinsic strength of a 5′ splice site and the calculated stability of its base pairing with U1 snRNA is consistent with recent results on the hierarchy for 5′ splice site preference *in vivo* (21). However, we have no valid explanation as to why GGA is sometimes effective for splicing, and sometimes not. It might be a case in which 'sequence context' proposed by Nelson and Green (7) affects splice site utilization.

As for the 3′ exon sequence, no particular requirement was shown in the splicing of the transcripts presented here. Mitchell *et al.* (22) reported that a mutant precursor carrying U as the first nucleotide of the 3′ exon was not properly spliced and led to skipping of the exon in Chinese hamster cells. We observed reduced splicing of a transcript carrying U as the first nucleotide of the 3′ exon, which was obtained by the accidental deletion of 1 nt of the 3′ exon during construction of the template (data not shown). Therefore, we presume that U at this position may reduce splicing efficiency and thereby induce alternative splicing in the *in vivo* splicing of a precursor with multiple introns. However, U at the first position of the 3′ exon should not generally inactivate the 3′ splice site since approximately 10% of vertebrate introns are followed by U (5) and most such precursors should be spliced.

We found no essential role for surrounding exon sequences in splicing. However, our results do not rule out the possibility of a specific or special sequence in the exon that influences the splicing efficiency. Furdon and Kole (8) found an inhibitory, or 'poison', sequence which consists of a long pyrimidine stretch. We found five CAGs, two of which were preceded with a pyrimidine rich sequence, in the HβF transcript upstream of N site. The suppressed splicing of the transcript carrying MI cassette at this N site is probably due to the nearby presence of these 3′ splice site like sequences.

A cryptic 5′ splice site (probably one 16 nt upstream of the authentic splice site in the Wt transcript) was activated by insertion of an intron (mI) at A site of the sense transcript (Fig. 4). Cryptic 5′ splice sites can usually be activated by a mutation of the nearby authentic splice site, and three such sites are known near the first 5′ splice site of the human β-globin gene (17, 20, 23). In our experiments, insertion of removable MI cassette at the same A site did not activate the cryptic site. These results could be explained by a hierarchy of the free energy increments for the base pairing of the possible splice sites with U1 snRNA: −4.9 kcal for mI insertion at A, −10.8 kcal for the cryptic splice site, and −11.3 kcal for the MI insertion at A (Fig. 3). Insertion of the intron (mI cassette) into B site, which is distant from the cryptic site and unfavorable position of splicing like A site, did not activate a cryptic site, either. Thus, our results show that a cryptic splice site can be activated by nearby insertion of an intron with an incompetent (weak) splice site. Recently, we found that annealing of a 2′-*O*- methyl oligoribonucleotide to the first 5′ splice site activated the nearby cryptic splice site in the β-globin transcript carrying an active alternative donor site at a distance (24).

Effect of intron position on splicing efficiency

The structural features of our transcripts allowed direct examination of the effect of intron position on splicing efficiency. Klinz and Gallwitz (13) examined this effect on yeast pre-mRNA splicing *in vivo* with insertion of an intron-carrying fragment into one of four positions. They found that a precursor with insertion at position 656 or 713 had a somewhat decreased splicing efficiency (70% or 60%) as compared to that with insertion at position 132 or 274 (95% or 90%). We did not observe such uniform dependence of splicing efficiency on the intron position

in the region of 14 nt to 370 nt from the 5′ end (MI series in Figs. 4 and 5). Our results are consistent with previous papers reporting that the variations of 5′ exon length (21–482 nt) did not affect the splicing efficiency *in vitro* (8, 9). A longer transcript or an *in vivo* system might show a position effect (13, 25).

Role of exon sequences in RNA stability

The β-globin mRNA was shown to be very stable in the cytoplasm with a half-life of over 17 h (26) while transiently expressed genes such as c-*fos* and c-*myc* have half-life values as low as 15 min (27, 28). It was shown that AU-rich sequences, consisting of one or more AUUUA motifs, in the 3′ non-coding region of many short-lived mRNA promote their metabolic instability (29). We showed that the sense transcript of the β-globin exon sequence was much more stable than the anti-sense transcript in the presence of the nuclear extract (Fig. 6). There are no AUUUA motifs in either transcript, and their overall pyrimidine contents are similar (49% vs. 50%). We also examined the stability of a transcript from the β-globin first intron fragment (HβI) from which both splice sites were deleted, and that of a corresponding anti-sense transcript (HβJ). Both intron derived HβI and HβJ RNA showed a degradation pattern similar to that of HβF (data not shown). In addition to this result, when dal cassette was inserted into HβI RNA, this transcript was unstable, but spliced, while that including DAI was rather stable and efficiently spliced (data not shown). Therefore, the relative stability of HβE RNA, whose degradation was significantly arrested after 1 h incubation, suggests presence of a signal in sense (HβE) RNA which serves to protect against attack by various nucleases rather than a destabilizing sequence in anti-sense (HβF) RNA. The nature of the stabilizing signal in HβE RNA is unknown.

Intron position and origin of a pre-mRNA intron

Of great interest is the evolutionary origin of an intron and RNA splicing. Considering the fact that the conservation of intron position (30, 31) is not observed for the majority of introns in eukaryotic genes, it can be argued that introns have been lost and/or gained during evolution (32–34). We showed that the *cis*-acting determinants essential for pre-mRNA splicing are localized in the intron itself plus 3 nt of the 5′ exon, rather than in the overall structure of pre-mRNA. It is remarkable that about half of the transcripts with insertion of intron alone (mI cassette) were efficiently spliced (Figs. 4 and 5). In the yeast *S. cerevisiae* and *S. pombe*, some inserted introns without any flanking exon sequence could be removed *in vivo* (35, 36). Thus, if a nuclear pre-mRNA intron integrates into another RNA, it can often be removed in a splicing reaction. In view of the requirement for splicing, our results indicate no strict limitations to position into which an intron is inserted. Some introns of nuclear pre-mRNA may have originated by reversal of mRNA splicing process, or they could have evolved from group II self-splicing intron which can integrate into an RNA by reverse self-splicing (37, 38).

ACKNOWLEDGMENTS

We are grateful to Dr. T. Maniatis for the gift of cloned human β-globin gene (pSP64-HβΔ6) and its cDNA (pSP64-HβΔ6-IVS1,2). We thank K. Mukai and S. Yamaguchi for their assistance with some of the plasmid DNA screening; Drs. A. Krainer, T. Tani, I. Mori and M. Ohara for critical reading of the manuscripts. A.M. was supported by a Fellowship of the Japan Society for the Promotion of Science for Japanese Junior Scientist. This work was aided by grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Green, M.R. (1986) *Ann. Rev. Genet.*, **20**, 671–708.
2. Krainer, A.R. and Maniatis, T. (1988) In Hames, B.D. and Glover, D.M. (eds.) Transcription and Splicing. IRL Press, Oxford, pp. 131–206.
3. Padgett, R.A., Grabowski, P.J., Konarska, M.M., Seiler, S. and Sharp, P.A. (1986) *Ann. Rev. Biochem.*, **55**, 1119–1150.
4. Mount, S.M. (1982) *Nucleic Acids Res.*, **10**, 459–472.
5. Ohshima, Y. and Gotoh, Y. (1987) *J. Mol. Biol.*, **195**, 247–259.
6. Reed, R. and Maniatis, T. (1986) *Cell*, **46**, 681–690.
7. Nelson, K.K. and Green, M.R. (1988) *Genes Dev.*, **2**, 319–329.
8. Furdon, P.J. and Kole, R. (1988) *Mol. Cell. Biol.*, **8**, 860–866.
9. Parent, A., Zeitlin, S. and Efstratiadis, A. (1987) *J. Biol. Chem.*, **262**, 11284–11291.
10. Furdon, P.J. and Kole, R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 927–931.
11. Eperon, L.P., Estiberio, J.P. and Eperon, I.C. (1986) *Nature*, **324**, 280–282.
12. Mayeda, A. and Ohshima, Y. (1988) *Mol. Cell. Biol.*, **8**, 4484–4491.
13. Klinz, F.-J. and Gallwitz, D. (1985) *Nucleic Acids Res.*, **13**, 3791–3804.
14. Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
15. Hattori, M. and Sakaki, Y. (1986) *Anal. Biochem.*, **152**, 232–238.
16. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) *Nucleic Acids Res.*, **11**, 1475–1489.
17. Krainer, A.R., Maniatis, T., Ruskin, B. and Green, M.R. (1984) *Cell*, **36**, 993–1005.
18. Ruskin, B., Krainer, A.R., Maniatis, T. and Green, M.R. (1984) *Cell*, **38**, 317–331.
19. Ruskin, B. and Green, M.R. (1985) *Nature*, **317**, 732–734.
20. Treisman, R., Orkin, S.H. and Maniatis, T. (1983) *Nature*, **302**, 591–596.
21. Lear, A.L., Eperon, L.P., Wheatley, I.M. and Eperon, I.C. (1990) *J. Mol. Biol.*, **211**, 103–115.
22. Mitchell, P.J., Urlaub, G. and Chasin, L. (1986) *Mol. Cell. Biol.*, **6**, 1926–1935.
23. Vidaud, M., Gattoni, R., Stevenin, J., Vidaud, D., Amselem, S., Chibani, J., Rosa, J. and Goossens, M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1041–1045.
24. Mayeda, A., Hayase, Y., Inoue, H., Ohtsuka, E. and Ohshima, Y. (1990) *J. Biochem.*, in press.
25. Greenspan, D.S. and Weissman, S.M. (1985) *Mol. Cell. Biol.*, **5**, 1894–1900.
26. Volloch, V. and Housman, D. (1981) *Cell*, **23**, 509–514.
27. Greenberg, M.E. and Ziff, E.B. (1984) *Nature*, **311**, 433–438.
28. Piechaczyk, M., Yang, J.-Q., Blanchard J.-M., Jeanteur, P. and Marcu, K.B. (1985) *Cell*, **42**, 589–597.
29. Shaw, G. and Kamen, R. (1986) *Cell*, **46**, 659–667.
30. Gilbert, W. (1985) *Science*, **228**, 823–824.
31. Zakut, R., Shani, M. Givol, D., Neuman, S., Yaffe, D. and Nudel, U. (1982) *Nature*, **298**, 857–859.
32. Perler, F., Efstratiadis, A., Lomedico, P., Gilbert, W., Kolodner, R. and Dodgson, J. (1980) *Cell*, **20**, 555–566.
33. Rogers, J. (1989) *Trends Genet.*, **5**, 213–216.
34. Sharp, P.A. (1985) *Cell*, **42**, 397–400.
35. Yoshimatsu, T. and Nagawa, F. (1989) *Science*, **244**, 1346–1348.
36. Gatermann, K.B., Hoffmann, A., Rosenberg, G.H. and Käufer N.F. (1989) *Mol. Cell. Biol.*, **9**, 1526–1535.
37. Augustin, S., Müller, M.W. and Schweyen, R.J. (1990) *Nature*, **343**, 383–386.
38. Mörl, M. and Schmelzer, C. (1990) *Cell*, **60**, 629–636.
39. Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T. and Turner, D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.