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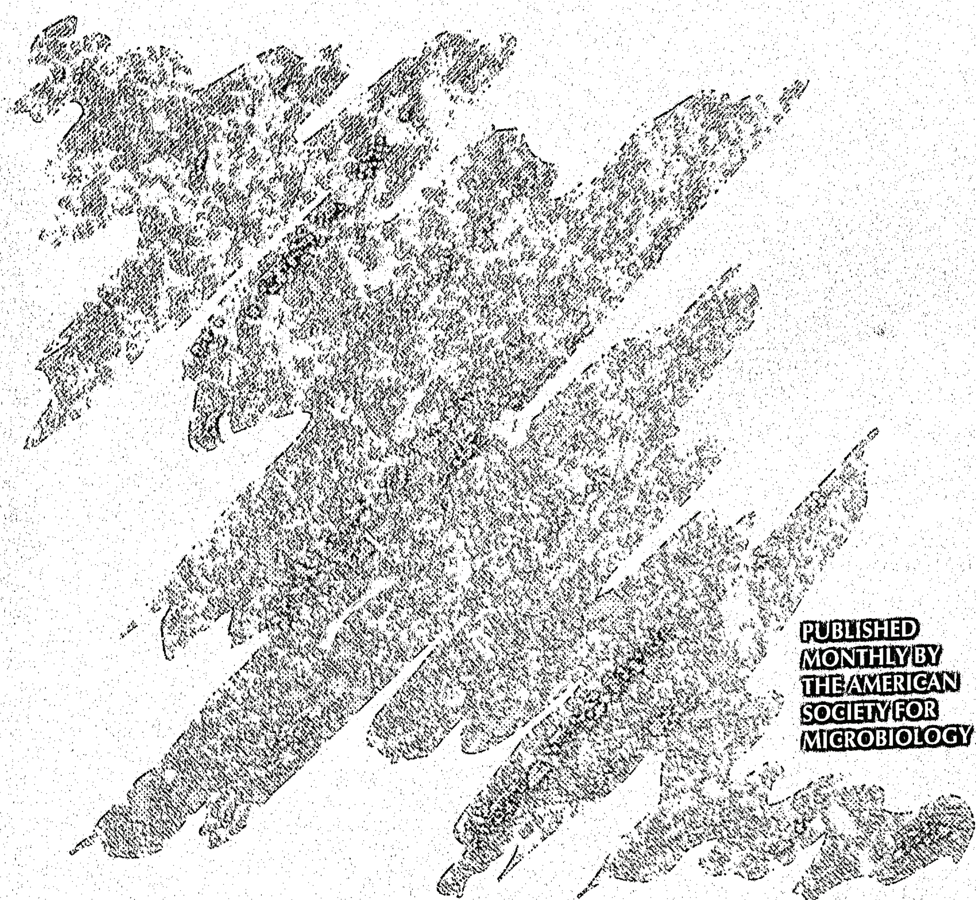

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Identification and Characterization by Antisense Oligonucleotides of Exon and Intron Sequences Required for Splicing

ZBIGNIEW DOMINSKI AND RYSZARD KOLE*

Department of Pharmacology and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

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Certain thalassemic human β-globin pre-mRNAs carry mutations that generate aberrant splice sites and/or activate cryptic splice sites, providing a convenient and clinically relevant system to study splice site selection. Antisense 2′-*O*-methyl oligoribonucleotides were used to block a number of sequences in these pre-mRNAs and were tested for their ability to inhibit splicing in vitro or to affect the ratio between aberrantly and correctly spliced products. By this approach, it was found that (i) up to 19 nucleotides upstream from the branch point adenosine are involved in proper recognition and functioning of the branch point sequence; (ii) whereas at least 25 nucleotides of exon sequences at both 3′ and 5′ ends are required for splicing, this requirement does not extend past the 5′ splice site sequence of the intron; and (iii) improving the 5′ splice site of the internal exon to match the consensus sequence strongly decreases the accessibility of the upstream 3′ splice site to antisense 2′-*O*-methyl oligoribonucleotides. This result most likely reflects changes in the strength of interactions near the 3′ splice site in response to improvement of the 5′ splice site and further supports the existence of communication between these sites across the exon.

Pre-mRNA splicing takes place within a large ribonucleoprotein complex termed the spliceosome. The specificity and accuracy of splicing are determined by the interactions of small nuclear ribonucleoprotein particles and protein components of the spliceosome with a number of pre-mRNA sequence elements in pre-mRNA, such as the branch point sequence, the polypyrimidine tract, and the 3′ and 5′ splice sites (reviewed in references 15, 21, and 29). In addition, exon sequences seem to contribute to the specificity of splicing (references 35, 41, and 49 and references therein). However, besides identification of a regulatory element in the female-specific exon of the doublesex pre-mRNA (17, 19, 30) and characterization of purine-rich motifs in exons from some other spliced transcripts (7, 12, 27, 45, 47, 49, 51), the involvement of exon sequences in splicing remains unclear.

In this work, we have used antisense 2′-*O*-methyl oligoribonucleotides (see reference 43 for a review) to study the function of several intron and exon sequences in pre-mRNA splicing. This approach stems from our recent report which showed that the binding of 2′-*O*-methyl oligoribonucleotides to the branch point or aberrant splice sites leads to the restoration of correct in vitro splicing of mutated β-globin pre-mRNAs identified in individuals with various forms of β-thalassemia (11). These oligonucleotides form strong duplexes with RNA which are resistant to RNase H and RNA unwinding activities. In consequence, they remain stably associated with the complementary regions in RNA, efficiently inhibiting the function of the targeted sequences. Antisense 2′-*O*-methyl oligoribonucleotides were originally used as sequence-specific probes to study the structure of small nuclear ribonucleoproteins and their interactions with the pre-mRNA substrate (43). They were also used to search for novel *cis*-acting sequence elements and possible scanning mechanisms during the splicing reaction (28).

We have targeted these oligonucleotides against human β-globin pre-mRNAs carrying mutations responsible for a number of β-globin thalassemia variants. The mutations generate new splice sites and/or activate cryptic ones, leading to aberrant splicing pathways (reviewed in reference 40). In the experiments described below, we took advantage of the fact that changes in the accumulation of the correctly spliced products relative to that of the aberrant ones provide a sensitive and internally controlled assay for monitoring interference with the activity of the sequence targeted by the antisense oligonucleotide. Using this approach, we have found that sequences upstream from the conserved branch point sequence as well as those within the exons are required for splicing and play a role in splice site selection. The experiments also showed that improving the 5′ splice site of the internal exon affects interactions at the upstream 3′ splice site.

MATERIALS AND METHODS

Plasmid construction. Previously described pSP64HβΔ6 (22) and its thalassemic derivatives cloned in the pSP64 vector were used in all studies. The pIVS1-β^{110} clone, carrying an A-to-G mutation in position 110 of the first intron, was constructed by subcloning an appropriate fragment from the original thalassemic clone (14a). The pIVS2-β^{705} clone was obtained by introducing a T-to-G mutation at position 705 of the large β-globin intron in the pIVS2 clone, as previously described (11, 25). A C-to-T mutation at position 654 and a TA-to-GT mutation at positions 657 and 658 of the large β-globin intron in the pIVS2 clone were introduced to create pIVS2-β^{654} and pIVS2-β^{654con} clones, respectively. The structures of all mutated constructs were confirmed by sequencing.

In vitro transcription and splicing. ^{32}P-labeled transcripts were synthesized as previously described (20) with SP6 RNA polymerase (Promega) and DNA templates cleaved within the second exon at either the *Bam*HI site (clones HβΔ6 and β^{110}), the *Pvu*II site (clone β^{705}), or the *Ase*I site (clones pIVS2, β^{654}, and β^{654con}). The preparation of nuclear extract from HeLa

* Corresponding author. Mailing address: University of North Carolina, Lineberger Comprehensive Cancer Center, CB#7295, Chapel Hill, NC 27599. Phone: (919) 966-1143. Fax: (919) 966-3015.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)	% GC
BPa	GUCAGUGCCUAUCA	50
BPb	GUGCCUAUCAGAAA	43
BPc	CCUAUCAGAAACCC	50
BPc/18	CCUAUCAGAAACCCAAGA	44
BPd	AUCAGAAACCCAAG	43
BPe	AAACCCAAGAGUCU	43
BPf	CAAGAGUCUUCUCU	43
Exa	ACCAGCAGCCUAAG	57
Exb	AGGGUAGACCACCA	57
Exc	UCUGGGUCCAAGGG	64
Exd	UCAAAGAACCUCUG	43
3′ss 579/14	UAUUGCCCUGAAAG	43
3′ss 579/18	CAUUAUUGCCCUGAAAGA	39
ExU1	GUAUCAUUAUUGCC	36
ExU2	UACAUUGUAUCAUU	21
ExU3	AGAGGCAUGAUACA	43
ExU4	AAUGGUGCAAAGAG	43
ExU5	UAUUCUUUAGAAUG	21
ExD1	CAGAAAUAUUUAUA	14
ExD2	AAUUUAUAUGCAGA	21
5′ss 705	CCUCUUACCUCAGUUAC	47
Int1	UAUUAGCAAUAUGA	21
Int2	UUGUAGCUGCUAUU	36
5′ss 652/18	GCUAUUACCUUAACCCAG	44
5′ss 652con/18	GCACUUACCUUAACCCAG	50
5′ss 652con/14	CACUUACCUUAACC	43

cells and in vitro splicing were performed as previously described (11, 22).

Synthesis and purification of oligonucleotides. 2′-*O*-methyl-ribonucleoside phosphoramidates (Glen Research, Sterling, Va.) were used for oligonucleotide synthesis in an Applied Biosystems synthesizer at the Lineberger Comprehensive Cancer Center. Oligonucleotides were purified by thin-layer chromatography (SurePure kit; U.S. Biochemicals), and their concentrations were determined spectrophotometrically at 260 nm. To ascertain the quality of oligonucleotides, the 5′-^{32}P-labeled compounds were analyzed by polyacrylamide gel electrophoresis.

Oligonucleotide treatment. In all experiments, 2′-*O*-methyl oligoribonucleotides were added to the reaction mixture together with the other components of the splicing reaction. The preannealing of oligonucleotides with transcript in the absence of nuclear extract has been shown not to increase their overall effect on splicing (11, 28). The extent of unspecific effects for each experiment was controlled by using oligonucleotides with no complementarity to the RNA substrate. Oligonucleotide 3′ss 579/14 was used to control unspecific effects during the splicing of pre-mRNAs containing the first β-globin intron, whereas oligonucleotide BPa was used during the splicing of pre-mRNAs containing the second intron. In some experiments, a mixture of randomly synthesized oligonucleotides was also used.

Sequences of oligonucleotides. The oligonucleotides used in this study are listed in 5′-to-3′ orientation in Table 1.

Data processing and analysis. All autoradiograms were captured by a DAGE MTI CCD72 video camera (DAGE, Michigan City, Ind.), and images were processed with NIH Image 1.47 and MacDraw Pro 1.0 software. The final figures were printed out on a Sony dye sublimation printer. Results were quantitated with NIH Image 1.47 software and expressed as percentages of correct product relative to the sums of correct and aberrant products. Values were adjusted to account for the higher number of ^{32}P-labeled C nucleotides in the longer, aberrantly spliced product. Averages obtained from several independent experiments and multiple exposures are presented.

RESULTS

Mapping the upstream boundary of the functional domain near the branch point sequence with β^{110} pre-mRNA substrate. A G-to-A point mutation at position 110 of the first intron in the β-globin gene (IVS1-β^{110}; Fig. 1B) creates a new 3′ splice site at position 109 in addition to the one located at position 130 of this intron. This mutation is responsible for a significant number of β-thalassemia cases in southeastern Europe, Cyprus, and Lebanon (40). The splicing of IVS1-β^{110} mutant pre-mRNA leads to predominant (approximately 90%) accumulation of a spliced product containing an additional 19 nucleotides of the intron sequence (Fig. 2A, lane 1). Remarkably, aberrant splicing of β^{110} pre-mRNA occurs via selection of the regular branch point at position 94 of the intron, whereas correct splicing (approximately 10% of the resultant mRNA) occurs via selection of the cryptic branch point at position 107 (54). As a result, mutations inactivating the regular branch point (38) or antisense oligonucleotides blocking it (11) stimulate the cryptic branch point and restore correct splicing in the β^{110} background.

In these studies, we used 2′-*O*-methyl oligoribonucleotides to determine the importance of sequences located upstream from the branch point region in the function of this splicing element. We have designed a series of 14-mers complementary to the region extending up to 32 nucleotides upstream from the branch point sequence (Fig. 1B). The ability of oligonucleotides to inhibit splicing at the aberrant 3′ splice site and at the same time to promote splicing at the correct 3′ splice site was taken as the measure of the function of the upstream sequences during the splicing of β^{110} pre-mRNA (11). Note that a concomitant switch in selection between the regular and cryptic branch points can be directly determined on autoradiograms because of the variable mobility of the corresponding lariats (54).

The results of in vitro splicing of β^{110} pre-mRNA carried out in the presence of 0.5, 2.0, and 10.0 μM oligonucleotide BPa, blocking all seven nucleotides of the branch point sequence, are shown in Fig. 2A. Consistent with the results of a previous report (11), the antisense 2′-*O*-methyl oligoribonucleotide targeted to this site leads to a change in the ratio between correct and aberrant products. A splicing reaction carried out at a 2 μM concentration of this oligonucleotide results in the accumulation of approximately 55% of the correct product (Fig. 2A, lane 3; see Fig. 2C for quantitation). Although 10 μM oligonucleotide BPa results in a marked decrease of the overall efficiency of the splicing reaction, it does not significantly modify the ratio between correct and aberrant products achieved at a 2 μM concentration (Fig. 2A, lane 4).

Figure 2B demonstrates the results of an in vitro splicing reaction of β^{110} pre-mRNA carried out in the presence of a 2 μM concentration of oligonucleotides directed upstream from the branch point adenosine. Similar to BPa, oligonucleotides BPb, -c, and -d restore correct splicing to 50 to 65% (Fig. 2B, lanes 2 to 4, respectively, and C). Note that oligonucleotides BPc and BPd hybridize immediately outside the conserved branch point sequence and four nucleotides upstream from this element, respectively. While oligonucleotide BPe, hybridized 13 nucleotides upstream from the branch point adenosine, still has some effect on the splicing pathway, oligonucleotide

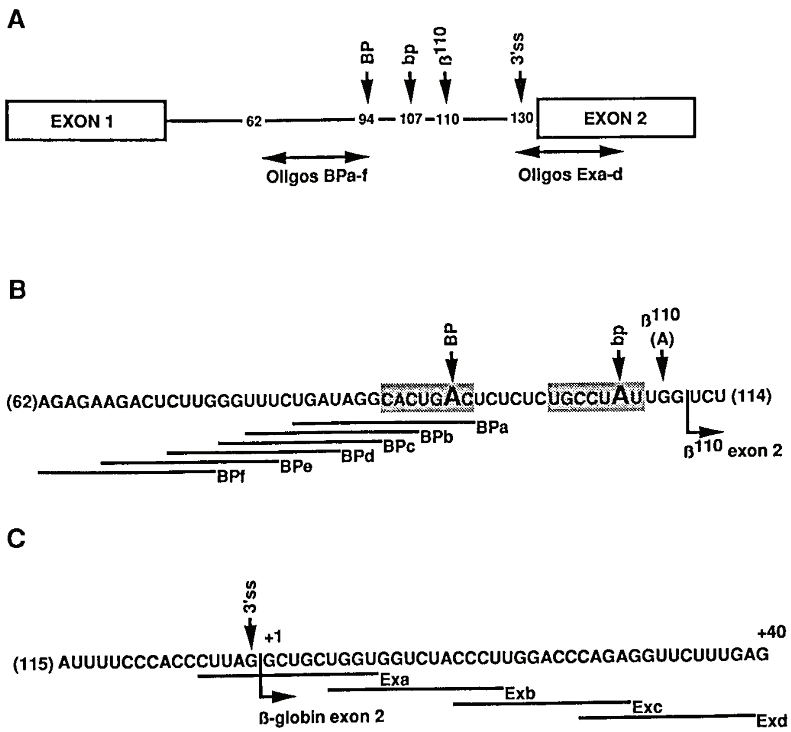

FIG. 1. (A) Structure of human β-globin pre-mRNA (not drawn to scale) containing the first intron (the line) and two flanking exons (boxes). The positions of the regular (BP) and cryptic (bp) branch point adenosines, β^{110} mutation, and the 3′ splice site (3′ss) are shown. The regions of pre-mRNA used as targets for 2′-*O*-methyl oligoribonucleotides are indicated by double-headed arrows. Exon 1 contains 154 nucleotides, and exon 2 contains 210 nucleotides. The exact sequence positions of oligonucleotides BPa to BPf (B) and Exa to Exd (C) are shown. The regular and cryptic branch point sequences (shaded boxes) and the aberrant (β^{110}) and correct 3′ splice sites are indicated.

BPf, targeted 5 nucleotides further upstream, seems to be ineffective. Consistently, oligonucleotide BPf, in contrast to the other probes tested, does not stimulate the formation of the lariat intermediate at the branch point adenosine at position 107, as indicated by the lack of a band migrating to the top of the gel (Fig. 2B, lane 6).

The involvement of exon sequences in the splicing of β^{110} pre-mRNA. To survey other regions of β-globin pre-mRNA for the existence of functional domains involved in splicing, we designed 14-mers complementary to the correct junction between the first intron and the second exon (oligonucleotide Exa) or to sequences extending downstream into the second exon (oligonucleotides Exb to -d) (Fig. 1C). For HβΔ6 pre-mRNA, oligonucleotide Exa partially overlaps with the intron sequence; in the case of β^{110} substrate, the same oligonucleotide has its target entirely within the aberrantly spliced second exon. Although the upstream end of the duplex formed between this oligonucleotide and its target in the β^{110} transcript is separated from the predominantly used aberrant 3′ splice site by 14 nucleotides, Exa oligonucleotide at a 2 μM concentration fully blocks splicing (Fig. 3, lane 3). This is in agreement with the observation that mutations disrupting the AG dinucleotide of the normal 3′ splice site inhibit the use of the aberrant splice site in β^{110} pre-mRNA (23, 54). An inhibitory effect on splicing is also observed in the presence of oligonucleotides Exb and Exc, which hybridize further downstream into the second exon, and, albeit to a lesser extent, in the presence of the most distal oligonucleotide, Exd, which hybridizes 44 nucleotides downstream from the β^{110} 3′ splice site (Fig. 3, lanes 4 to 6). The control noncomplementary oligonucleotide does not significantly affect the efficiency of the splicing reaction (Fig. 3, lane 7). A pattern of inhibition similar to that described above was obtained when each of the oligonucleotides Exa, -b, -c and -d was added to the splicing reaction with normal β-globin pre-mRNA (data not shown).

The involvement of exon sequences in the splicing of the β^{705}-globin pre-mRNA. The results of Zhuang and Weiner (54) suggested that the splicing of β^{110} pre-mRNA may represent a special case in which the AG dinucleotide located inside the exon is required for splicing at the upstream 3′ splice site. To confirm that exon involvement in splicing is not limited to only one type of substrate, we analyzed the effects of a series of antisense oligonucleotides (Fig. 4B and C) on the splicing of thalassemic β^{705} pre-mRNA. A T-to-G mutation at position 705 of the large β-globin intron creates an additional 5′ splice site and activates a cryptic 3′ splice site at position 579 of this intron (8). The incorrect splicing pathway resulting from the utilization of both splice sites leads to the incorporation of nucleotides 580 to 705 of the intron into the spliced product and the accumulation of significant amounts of the 577-nucleotide aberrant RNA in addition to the correct product (Fig. 4A). Changes in the ratio between correct and aberrant products provide a sensitive method for measuring the sequence-specific effects of the oligonucleotides used.

Figure 5A shows the complete pattern of in vitro splicing of the β^{705} substrate. Quantitative analysis indicates that the ratio

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