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Sensitivity of splice sites to antisense oligonucleotides in vivo

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ABSTRACT

A series of HeLa cell lines which stably express β-globin pre-mRNAs carrying point mutations at nt 654, 705, or 745 of intron 2 has been developed. The mutations generate aberrant 5′ splice sites and activate a common 3′ cryptic splice site upstream leading to aberrantly spliced β-globin mRNA. Antisense oligonucleotides, which in vivo blocked aberrant splice sites and restored correct splicing of the pre-mRNA, revealed major differences in the sensitivity of these sites to antisense probes. Although the targeted pre-mRNAs differed only by single point mutations, the effective concentrations of the oligonucleotides required for correction of splicing varied up to 750-fold. The differences among the aberrant 5′ splice sites affected sensitivity of both the 5′ and 3′ splice sites; in particular, sensitivity of both splice sites was severely reduced by modification of the aberrant 5′ splice sites to the consensus sequence. These results suggest large differences in splicing of very similar pre-mRNAs in vivo. They also indicate that antisense oligonucleotides may provide useful tools for studying the interactions of splicing machinery with pre-mRNA.

Keywords: alternative splicing, pre-mRNA splicing, splicing factors, splicing mutations

INTRODUCTION

Pre-mRNA splicing takes place in a multicomponent complex, the spliceosome, which forms *de novo* during each splicing event and disintegrates to its constituent components after the splicing reaction has been completed. In the early steps, the pre-mRNA sequences at the 5′ splice site and at the branch point are base paired with the 5′ end of small nuclear RNA U1 (U1 snRNA) and the internal portion of U2 snRNA, respectively. These RNAs are each complexed with a number of proteins forming ribonucleoprotein particles (snRNPs). In addition to U1 and U2, the functional spliceosome includes U4/U6.U5 triple snRNP and a number of other protein splicing factors. Altogether, the relatively short sequences at the splice sites and the branchpoint interact with or are in proximity to five snRNAs and at least seventy proteins (reviewed in Moore et al., 1993; Krämer, 1996; Reed, 1996; Will & Lührmann, 1997; Staley & Guthrie, 1998).

The structure of the spliceosome described above appears to be only rudimentary and for multi-intron pre-mRNAs may be even more complex. Efficient recognition of internal, especially alternative, splice sites is enhanced by additional sequences, so called exonic or intronic splicing enhancers. On the other hand, partial, that is, inefficient splicing of retroviral genomic RNAs requires suboptimal splice sites and/or splicing silencers. In either case these pre-mRNA sequences are bound by protein factors that in turn interact with components of the canonical spliceosome, for example, U2AF or U1 70K proteins. The splicing factors that are responsible for the enhancer/silencer activity usually belong to a family of SR-proteins that share an arginine-serine domain as well as RNA binding domains (Manley & Tacke, 1996; McNally & McNally, 1996; Valcarcel & Green, 1996; Hertel et al., 1997; Wang & Manley, 1997). SR-proteins appear to be part of a larger group of splicing factors in which arginines may alternate with amino acids other than serines (Blencowe et al., 1995; Neugebauer et al., 1995). Thus, the components of the spliceosome may be even more numerous than was initially believed. Moreover, pre-mRNA sequences that interact with splicing factors extend well beyond the splice sites and the branch point.

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There is compelling evidence that exons are the sequence elements initially recognized in multi-intron pre-mRNAs (Berget, 1995). Binding of U1 snRNP to the downstream 5′ splice site and of U2 snRNP to the branchpoint in the upstream intron is stabilized by a series of RNA–protein and protein–protein interactions across the exon. For example, purine rich splicing enhancers bind SR-proteins such as ASF/SF2 or SC35, which in turn bridge the exon and stabilize the splicing complex by protein–protein interactions with U1 70K subunit and U2AF (Manley & Tacke, 1996; Hertel et al., 1997). Exon recognition is promoted not only by purine rich enhancers but also by a variety of other sequences (Dominski & Kole, 1994a; Staknis & Reed, 1994; Tian & Kole, 1995; Coulter et al., 1997) suggesting that numerous protein factors, presumably members of the SR and SR-like families, may be involved in exon bridging. Hence, as judged mostly from in vitro experiments, during splicing of various exons the composition of the spliceosomes and/or their interactions with pre-mRNA may differ significantly.

We have investigated point mutations at positions 654, 705, and 745 of intron 2 of the human β-globin gene (IVS2-654, IVS2-705, IVS2-754) that create additional 5′ splice sites within the intron at or in close proximity to the mutation site (Treisman et al., 1983; Cheng et al., 1984; Dobkin & Bank, 1985). Each mutation also activates a cryptic 3′ splice site at position 579 resulting in the insertion of an aberrant, exon-like sequence of, respectively, 73, 126, or 165 nt into the spliced β-globin mRNA (Fig. 1A). The insertions generate a stop codon (Fig. 1B) preventing proper translation of the mRNA and causing β-thalassemia, a genetic disorder (Schwartz & Benz, 1995). Even though the correct splice sites still exist in all three mutants, the splicing pathways are shifted to aberrant ones because of the creation of internal aberrant exons by the thalassemic mutations.

In nuclear splicing extracts, blocking of the aberrant 5′ splice sites or the cryptic 3′ splice site activated by the IVS2-654 and -705 mutations in β-globin pre-mRNA with 2′-*O*-methyl-oligoribonucleotides reversed aberrant and restored correct splicing. The concentrations of the oligonucleotides required for 50% correction of splicing (EC_{50}) in both 654 and 705 mutated pre-mRNA was approximately 0.1 and 0.2 μM for the 5′ and 3′ splice sites, respectively, indicating that the sensitivity of the splice sites to the oligonucleotides was quite similar (Dominski & Kole, 1993). The results presented below show that the sensitivity of the same sequences to the oligonucleotides in vivo, in HeLa cells constitutively expressing thalassemic β-globin pre-mRNAs, differs markedly in the IVS2-654, -705, and -745 mutants.

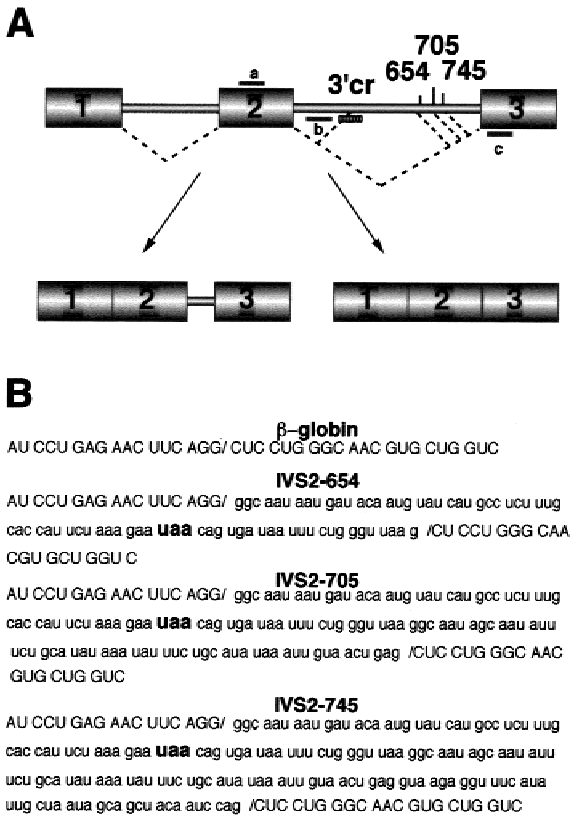

FIGURE 1. **A**: Splicing of human β-globin IVS2 mutant pre-mRNAs. Numbered boxes: exons; shaded lines: introns. Dashed lines indicate both correct and aberrant splicing pathways. The aberrant 5′ splice sites created by IVS2-654, -705, and -745 mutations and the cryptic 3′ splice site activated upstream are indicated. Shaded bar: oligonucleotide antisense to the aberrant 3′ splice site. Solid lines marked a, b, and c indicate primers used in the RT-PCR reaction. **B**: Sequence of β-globin and mutant spliced mRNAs. For β-globin mRNA a fragment of the sequence across the splice junction between exons 2 and 3 is shown in upper case. Sequences for mutant RNAs include aberrant exons (lower case). The stop codon generated by the three mutations is shown in bold.

RESULTS

To test the in vivo effects of antisense oligonucleotides, HeLa based cell lines stably expressing human thalassemic β-globin genes IVS2-654, -705, and -745 driven by the CMV promoter were generated by cotransfection with plasmids carrying the appropriate globin gene and the neomycin resistance marker. The stably transfected cells were treated with complexes of Lipofectamine and 17 or 18-mer 2′-*O*-methyl-oligoribonucleoside phosphorothioates (Table 1) targeted to the aberrant splice sites resulting from a given mutation (see Sierakowska et al., 1996 and Materials and Methods).

Correction of splicing of IVS2-654 pre-mRNA by antisense oligonucleotide, ON-654

The results of treatment of IVS2-654 HeLa cells with oligonucleotide ON-654, targeted to the aberrant 5′ splice site created by the mutation, are illustrated in Figure 2. Analysis of total cellular RNA by RT-PCR (see Materials and Methods) showed that the oligonucleotide

TABLE 1. Antisense oligonucleotides.

Oligonucleotide	Sequence	%GC	*Tm* (°C)[a]
ON-654	GCU AUU ACC UUA ACC CAG	44	46
ON-654con	GCA GUU GCC UUA ACC CAG	56	50
ON-705	CCU CUU ACC UCA GUU ACA	44	46
ON-745	GAA UGG UAC CUG GAU UGU	44	46
ON-3′CR	CAU UAU UGC CCU GAA AG	41	42

[a] *Tm* values were calculated for oligodeoxynucleotides and not for the 2′-*O*-methyl phosphorothioate derivatives. Nevertheless, they indicate the relative hybridization potential of the modified oligonucleotides used in this study.

restored correct splicing of the β-globin mRNA in a dose dependent manner (Fig. 2, lanes 2–6). At 0.2 μM extracellular concentration of the oligonucleotide, the maximum amount (approximately 48%, see Materials and Methods for quantitation of the results) of the β-globin RNA was converted to the correctly spliced form (Fig. 2, lane 4). Note that the maximum uptake of the oligonucleotide into the cell requires a specific charge ratio between the negatively charged oligonucleotide and the positively charged Lipofectamine (Hawley-Nelson et al., 1993; Bennett, 1998). This charge ratio was exceeded at 0.4 and 0.6 μM concentration of the oligonucleotide leading to a decrease in the correction of splicing (Fig. 2, lanes 5 and 6). However, the design of this experiment allows for determination of the true maximal effect under given experimental conditions. The effects are strictly sequence specific since a number of control oligonucleotides such as those with random sequence, mismatch, or targeted to regions of the intron distant from the splicing elements were ineffective (Sierakowska et al., 1996; Kang et al., 1998, and data not shown).

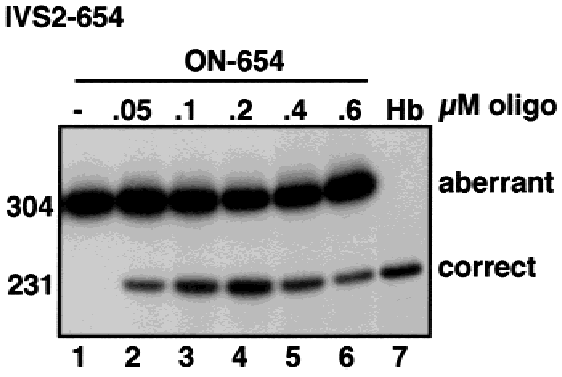

FIGURE 2. Correction of splicing of IVS2-654 pre-mRNA in HeLa cells by antisense oligonucleotide, ON-654, targeted to the aberrant 5′ splice site. Analysis of total RNA by RT-PCR with forward and reverse primers a and c, respectively (see Materials and Methods and Fig. 1A). Unless otherwise indicated these primers are used in subsequent figures. Lane 1: untreated HeLa cells; lanes 2–6: IVS2-654 HeLa cells treated with increasing concentrations of the oligonucleotide (in μM, indicated at the top); Lane 7 (Hb): RNA from human blood. In this and subsequent figures the numbers on the left indicate the size, in nucleotides, of the RT-PCR products representing the aberrantly (304) and correctly (231) spliced RNAs. Note that RT-PCR was carried out in the presence of a[^{32}P]-ATP and that the adenosine nucleotide content in the aberrantly spliced product is 1.57 times that of the correctly spliced one. Thus, the degree of correction is higher than it appears from the autoradiogram (see Materials and Methods).

Correction of splicing of IVS2-705 and IVS2-745 pre-mRNAs by antisense oligonucleotides

Correction of splicing of HeLa cell line IVS2-705 pre-mRNA with ON-705 oligonucleotide was much easier to achieve than that in the IVS2-654 cell line with ON-654. Correctly spliced β-globin mRNA was easily detectable at 1 nM ON-705, (Fig. 3A, upper panel, lane 3) and at 50–200 nM, correction of splicing was essentially complete (Fig. 3A, lanes 6 and 7). As in the IVS2-654 experiment, higher concentrations of the oligonucleotide led to a decrease in correction of splicing (Fig. 3A, lanes 9 and 10). The sequence specificity of these effects was shown by the lack of correction of splicing in cells treated with a random sequence oligonucleotide (ON-ran) in a broad range of concentrations (Fig. 3A, lower panel) or with mismatched oligonucleotides (data not shown).

To ascertain that the RT-PCR assay does not overestimate the concentration of the newly generated correctly spliced β-globin mRNA, the total protein from oligonucleotide-treated IVS2-705 HeLa cells was assayed by immunoblotting with an anti-human hemoglobin antibody (see Materials and Methods). In agreement with the results shown in Figure 3A, upper panel, high levels of the β-globin polypeptide were detected in cells treated with 50–400 nM ON-705 (Fig. 3B, lanes 7–9). β-Globin was easily detected even at 3–10 nM ON-705 (Fig. 3B, lanes 5 and 6). Thus, generation of the globin protein confirms that correction of splicing of IVS2-705 pre-mRNAs takes place at a significantly lower concentration of antisense oligonucleotides than that of IVS2-654 pre-mRNA (see also Fig. 1 in Sierakowska et al., 1996).

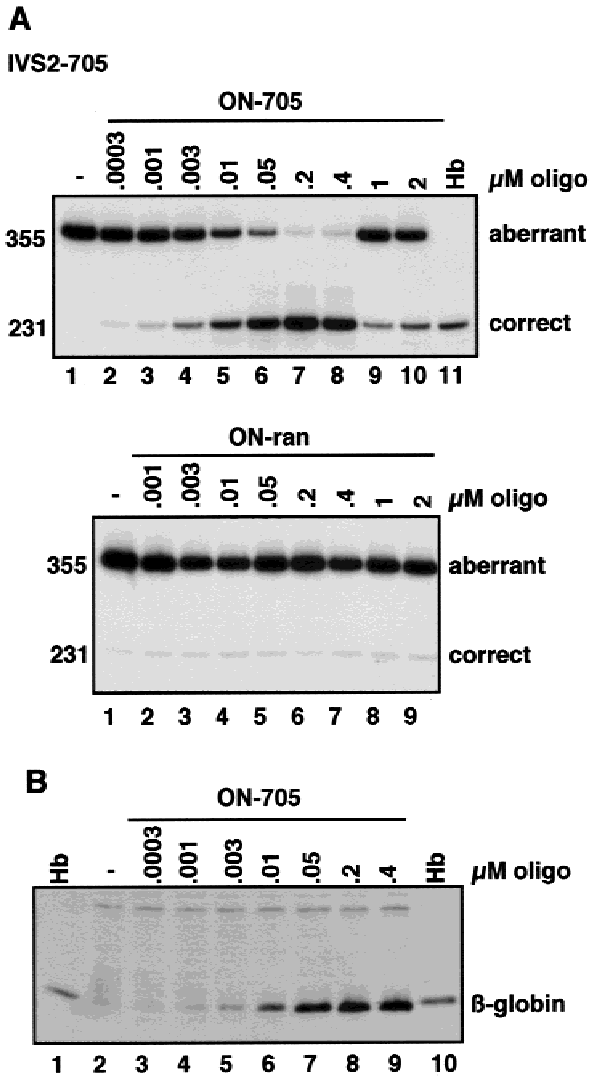

FIGURE 3. **A**: Correction of splicing in IVS2-705 HeLa cell line. Upper panel: treatment of cells with ON-705 oligonucleotide. Lower panel: treatment of IVS2-705 HeLa cells with random sequence oligonucleotide (ON-ran) as sequence specificity control. All other designations are as in Figure 2. **B**: Restoration of β-globin expression by ON-705 oligonucleotide in IVS2-705 HeLa cells. Immunoblot of total cellular protein probed with anti-human hemoglobin antibody. Lanes 1 and 10: human globin (Hb) (Sigma) used as a marker; lane 2: untreated cells; lanes 3–9: cells treated with increasing concentrations (in μM, indicated at the top) of ON-705.

Restoration of correct splicing of IVS2-745 pre-mRNA was even more efficient than in the other two mutants. At 10 nM ON-745 oligonucleotide, only correct β-globin mRNA was detected (Fig. 4, lane 3). Interestingly, although the aberrantly spliced RNA was no longer detectable, the level of β-globin mRNA increased in a dose dependent manner at 30–300 nM ON-745 (Fig. 4, lanes 5–7). A likely interpretation of this result is that the stability of the correct β-globin mRNA is higher than that of its aberrantly spliced 745 counterpart and therefore the product of correct splicing of IVS2-745 pre-mRNA accumulates at a higher steady state level.

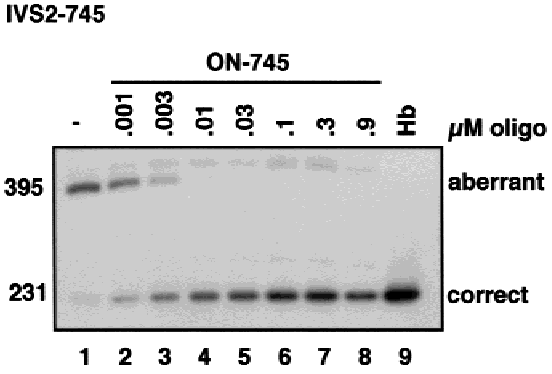

FIGURE 4. Correction of splicing in IVS2-745 HeLa cell line. Treatment of cells with ON-745. All designations are as Figure 2.

Analysis of IVS2 mutants and oligonucleotides

Quantitation of the results shown in Figures 2–4 and of additional experiments and the linear regression analysis of the oligonucleotide concentration shows that the EC_{50} of the oligonucleotides targeted to the three aberrant 5′ splice sites differs by up to 115-fold (Table 2 and Materials and Methods). Several lines of evidence indicate that the difference in the sensitivity of the targeted splice sites is an intrinsic property of the spliced pre-mRNAs and is not due to variations of the oligonucleotides and/or the cell lines. The levels of aberrantly spliced RNAs decrease significantly in the order IVS2-654, -705, -745 (compare lane 1 in Figs. 2, 3A, and 4) suggesting that expression of the thalassemic genes could be different in the three cell lines. Consequently, the ratio of the oligonucleotide to the pre-mRNA target could be much higher in IVS2-705 and -745 than in IVS2-654 cells. This higher ratio would favor hybridization of the oligonucleotides, promoting correction of splicing. However, the level of β-globin pre-mRNA, assayed by RT-PCR of total cellular RNA, was found to be lowest in the IVS2-654 cell line, intermediate in -705, and the highest in -745 (Fig. 5, upper panel, lanes, 2, 3, and 5; the construct IVS2-654con, lane 4, is discussed in the subsequent section). A control PCR reaction, with the reverse transcription step omitted, shows that these results are not due to genomic DNA, a possible contaminant of the RNA preparation (Fig. 5, lower panel). This indicates that correction of splicing of IVS2-654 pre-mRNA was the least efficient in spite of the most favorable ratio of the antisense oligonucleotide to its target (see also Discussion).

Although the 18-mer oligonucleotides ON-654, -705, and -745 differ in sequence, their hybridization potential, as reflected by their GC content and *Tm* values (Table 1), is identical and hence unlikely to be responsible for the observed differences in correction of splicing. The secondary and/or tertiary structure of the pre-mRNAs around the targeted splice sites is also unlikely to differentially affect access of the oligonucle-

TABLE 2. Effective concentration of antisense oligonucleotides targeted to IVS2 mutant pre-mRNAs.

			EC_{50} (nM)[b]	
Pre mRNA	5′ splice site	U1[a]	5′[c]	3′cr
IVS2-654	CUGGGUUAAG/GUAAUAGC		115	1,560
	|| |||| |			
U1	GUC/CAUUCAUA	7/11		
IVS2-705	UGUAACUGAG/GUAAGAGG		4	31
	|| ||||| |			
U1	GUC/CAUUCAUA	8/11		
IVS2-745	UACAAUCCAG/GUACCAUU		1	2
	||| ||| |			
U1	GUC/CAUUCAUA	7/11		
IVS2-654con	CUGGGUUAAG/GUAAGUGC		1,420	ND[d]
	|| |||||||			
U1	GUC/CAUUCAUA	9/11		
IVS2-705con	UGUAACUGAG/GUAAGUGG			133
	|| ||||||			
U1	GUC/CAUUCAUA	9/11		
	3′ cryptic splice site			
All RNAs	UUUCAG/GGCAAUAAUG			

[a] As a measure of splice site strength, the number of nucleotides within the splice site that base pair with the 5′ end of U1 snRNA is shown. The relevant fragment of U1 sequence is shown under the splice site sequence.

[b] Concentration of an oligonucleotide that restored 50% of correct splicing was calculated as described in Materials and Methods.

[c] 5′ denotes oligonucleotides targeted to the aberrant 5′ splice sites of the respective IVS2 mutants, that is, ON-654 targeted to IVS2-654 pre-mRNA, ON-705 targeted to IVS2-705 pre-mRNA, and so forth. 3′cr denotes ON-3′cr oligonucleotide targeted to the cryptic 3′ splice site common to all IVS2 mutants. The sequences of the oligonucleotides are listed in Table 1.

[d] Correction of splicing was not detectable.

otides. Computer analysis (Jaeger et al., 1989; Wisconsin Package, V. 9.1) of the folding of IVS2-654, -705, and -745 introns, which vary only by single point mutations, did not reveal any obvious differences (not shown).

Splicing of thalassemic pre-mRNA in two different clones of HeLa IVS2-654 and three different clones of HeLa-705 cells showed similar differences in sensitivity to ON-654 and ON-705 nucleotides, respectively, as well as to the 3′-cryptic splice site oligonucleotide common to all three mutants (data not shown, see also below). These differences were retained in two independent clones of IVS2-654 and two of IVS2-705 cell lines based on erythroid, K-562 cells (L. Gorman and R. Kole, unpubl.). Likewise, the differences in sensitivity between IVS2-654, -705, and -745 were observed in cells transfected with U7 snRNAs targeted to the aberrant splice sites (Gorman et al., 1998 and unpubl. data). Furthermore, 3T3 IVS2-654 cells (Sierakowska et al., 1996) showed sensitivity to ON-654 characteristic of the HeLa and K562 IVS2-654 cells. These observations exclude the possibility that the selected clonal cell lines might have differed in their ability to uptake the oligonucleotides or in other properties that affected the results but which were unrelated to the splicing mutations in the β-globin pre-mRNA.

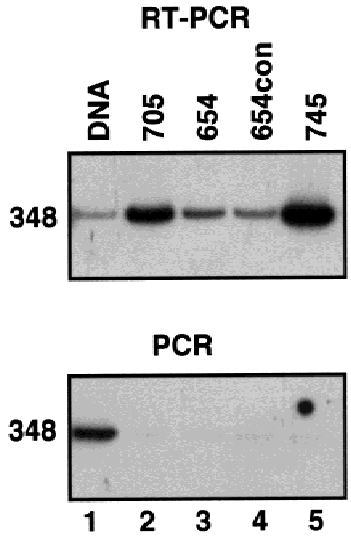

FIGURE 5. Expression of mutant IVS2 pre-mRNAs in HeLa cell lines. Total RNA from IVS2-705 (lane 2), -654 (lane 3) -654con (lane 4), and -745 (lane 5) cells was subjected to RT-PCR (upper panel) with forward and reverse primers a and b, respectively (see Materials and Methods and Fig. 1A). In lane 1, as size marker control, DNA from a plasmid carrying a human β-globin gene was also subjected to RT-PCR. In lower panel, the reverse transcription step was omitted and the samples were subjected to PCR only. Only traces, if any, of contaminating DNA were detected in the RNA samples.

Targeting the cryptic 3′ splice site with ON-3′cr

The fact that the same cryptic splice site is activated by IVS2-654, -705, and -745 mutations allows for designing a single oligonucleotide that should modify the splicing pathways in all the three pre-mRNAs. This approach eliminates any possible effects of the variations in oligonucleotide and target sequences inherent in the above experiments. Thus, the extent of splicing correction for each pre-mRNA must reflect the sensitivity of the target sequence to the oligonucleotide.

Figure 6 shows that in spite of the fact that the same 17-mer oligonucleotide (ON-3′cr, Table 1) binds to the same target sequence, the extent of correction of splicing differs significantly for each of the mutant RNAs and that the results follow the trend seen for the oligonucleotides targeted to the aberrant 5′ splice sites. The HeLa IVS2-654 cell line was highly resistant to treatment with ON-3′cr oligonucleotide and only minor quantities of correctly spliced β-globin mRNA could be detected at 0.1 and 0.2 μM oligonucleotide (Fig. 6A, lanes 3 and 4, respectively). In contrast, treatment of the HeLa IVS2-705 cell line with ON-3′cr corrected splicing close to 70% at 0.1 μM (Fig. 6B, lane 7). Correction of splicing of IVS2-745 pre-mRNA by ON-3′cr was very effective and was complete at 0.03 μM oligonucleotide (Fig. 6C, lane 5). Remarkably, EC_{50} of ON-3′cr varies from 1.5 μM for the IVS2-654 mutant to 2 nM for IVS2-745, a range of almost three orders of magnitude (Table 2).

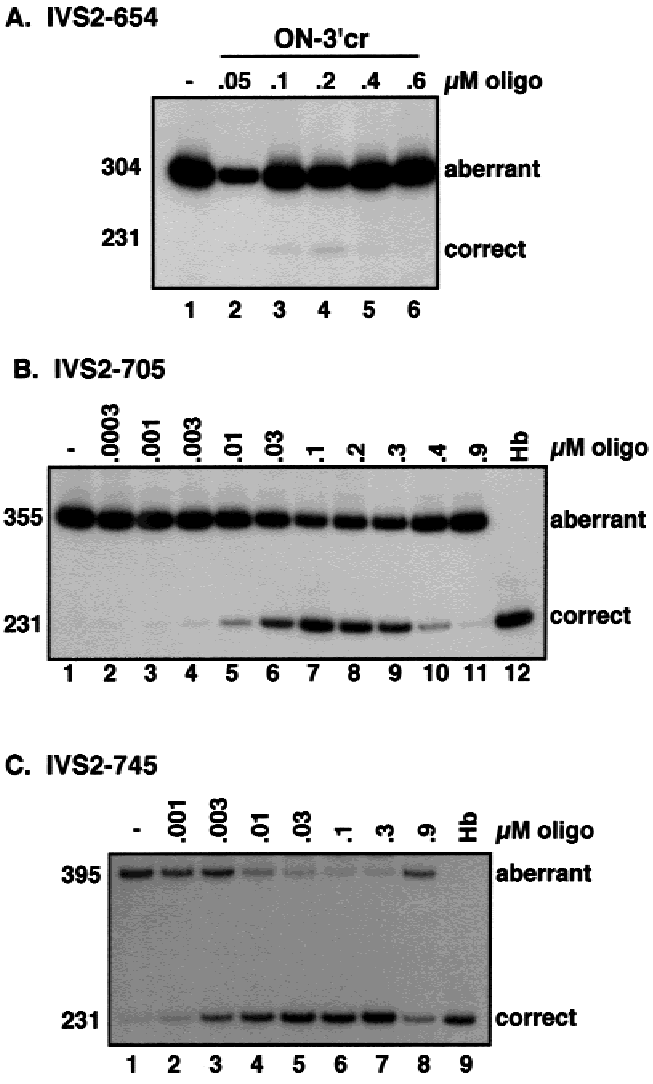

FIGURE 6. Treatment of IVS2-654 (**A**), -705 (**B**), and -745 (**C**) HeLa cell lines with ON-3′cr oligonucleotide. This oligonucleotide targets the 3′ cryptic splice site common to the three mutant pre-mRNAs. All designations are as in Figure 2.

Splice site strength versus sensitivity to oligonucleotides

Analysis of the data in Table 2 for IVS2-654, -705, and -745 may suggest, paradoxically, that sensitivity of the 5′ splice sites to the oligonucleotides is not related to the predicted splice site strength. Judging by the potential of the various 5′ splice sites to base pair with U1 snRNA (Table 2) or by calculation (Senapathy et al., 1990) of their match to the consensus splice site sequence (data not shown), the IVS2-654 5′ splice site is expected to be weaker than IVS2-705 and of equal strength with IVS2-745. Nevertheless, the IVS2-654 splice site was found to be 29- and 115-fold less sensitive than its -705 and -745 counterparts, respectively (Table 2). To investigate the mechanisms that may govern the response of the splice sites to oligonucleotides, we have modified the noncanonical but relatively inaccessible IVS2-654 5′ splice site (AAG/GUAA**UA**) to a consensus splice site (AAG/GUAA**GU** in IVS2-654con pre-mRNA, Table 2). The near-perfect but sensitive IVS2-705 5′ splice site was also converted to a consensus sequence IVS2-705con (GAG/GUAAG**A** to GAG/GUAAG**U,** respectively).

Treatment of the IVS2-654con HeLa cell line with 0.2 and 0.4 μM ON-654con led to barely detectable correction of splicing (Fig. 7, lanes 9 and 10, respec-

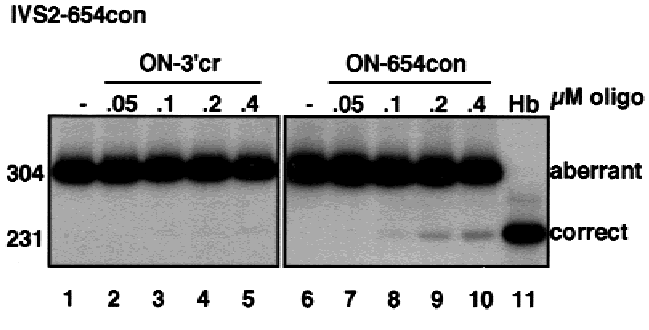

FIGURE 7. Poor accessibility of the aberrant splice sites in IVS2-654con pre-mRNA. HeLa cells expressing IVS2-654con pre-mRNA were treated with ON-3′cr (lanes 2–5) or ON-654con (lanes 7–10). Only minimal correction of splicing was observed in lanes 9 and 10. Lanes 1 and 6: untreated cells; lane 11: RNA from human blood.

tively) while ON-3′cr failed to correct splicing at any of the tested concentrations of the oligonucleotide (Fig. 7, lanes 2–5). Since the level of aberrantly spliced β-globin RNA was high, it raised the possibility that strong expression of the IVS2-654con pre-mRNA might have led to an unfavorable ratio of the target pre-mRNA to the oligonucleotides. However, analysis of the RNA shown in Figure 5 (lane 4, upper panel) indicates that the cellular level of IVS2-654con pre-mRNA was actually lower than that in the other cell lines. Thus, the change in the 5′ splice site, which increased its match to the consensus and complementarity to U1 snRNA, must have been responsible for decreased sensitivity of this sequence to the oligonucleotide. Interestingly, the change in the IVS2-654 5′ splice site eliminated correction of splicing by ON-3′cr, targeted to the upstream 3′ splice site, indicating that there is interplay between the splice sites.

Modification of the relatively strong IVS2-705 splice site to a consensus sequence in IVS2-705con construct also supports the above conclusions. Although, in contrast to IVS2-654con cells, correct β-globin mRNA was easily detected in IVS2-705con HeLa cells treated with ON-3′cr oligonucleotide, its amount was decreased in comparison to IVS2-705 cells. The EC_{50} of ON-3′cr for IVS2-705con was 133 nM, approximately fourfold higher than for IVS2-705 cells (31 nM, Table 2). Clearly, changes in the 5′ splice site affect not only its sensitivity to the oligonucleotides but also that of the upstream 3′ splice site. The differences between the IVS2-654 and IVS2-705 cell lines and their consensus derivatives suggest that additional sequence elements of the aberrant internal exons modulate splice site sensitivity (see Discussion).

DISCUSSION

The IVS2-654, -705, and -745 mutant pre-mRNAs differ only by single nucleotides and functionally share the upstream intron, the 3′ splice site and the first 75 nt of the aberrant exon. In the first two mutants the aberrant exon is fully included in the spliced message whereas in the third one, its inclusion is partial but still quite efficient. However, application of antisense oligonucleotides for in vivo probing of splicing pathways indicates that in spite of their sequence and functional similarities, the mutated pre-mRNAs show major differences in their interactions with the oligonucleotide probes. This is best illustrated by the 750-fold difference in the response of the 3′ splice site in IVS2-654 versus IVS2-745 pre-mRNAs to the antisense oligonucleotide, ON-3′cr. The experiments presented in this report show that these differences are due to the nature of splicing mutations in β-globin pre-mRNA and not to the variability in cellular background or in oligonucleotide uptake or efficacy. Two possible mechanisms may be responsible for this phenomenon: the differences in the rates of pre-mRNA splicing or in the accessibility of the splice sites to the oligonucleotides.

The levels of mutant pre-mRNAs, the targets for oligonucleotides, are low in IVS2-654 and IVS2-654con cell lines, in which correction of splicing is difficult, and higher in the easily correctable IVS2-705 and IVS2-745 cell lines. Assuming that the rate of β-globin RNA transcription, driven in all cell lines by the CMV promoter, is similar, the low levels of poorly correctable pre-mRNAs suggest that these RNAs are spliced at a high rate. If the hybridization of the oligonucleotides to the target sequences were much slower than the rate of pre-mRNA splicing, blocking of the aberrant splice sites by the oligonucleotides, and, in consequence, the shift of the splicing pathway from aberrant to correct might be disfavored. If the rates of aberrant splicing and oligonucleotide hybridization were not significantly different, however, then poor correction of efficiently spliced pre-mRNAs would suggest that their splice sites were not accessible to the oligonucleotides, presumably because of steric hindrance of strongly bound splicing factors. Note that in the latter scenario, the low levels of pre-mRNA result in a high ratio of oligonucleotides to their targets, which could be expected to promote hybridization and splicing correction. Although the experiments do not distinguish between the kinetic or steric hindrance mechanisms, both must be due to the differences in the interactions of the splicing factors with the splice sites and/or other sequence elements in the β-globin pre-mRNAs. It follows that the effective concentration of the oligonucleotides (EC_{50}) necessary for correction of splicing provides a quantitative, although indirect, measure for the strength of binding of the elements of the spliceosome to their target sequences.

It appears that the strength of the aberrant 5′ splice sites and the distance between the 3′ and the 5′ splice sites flanking the internal exons determine the differences in EC_{50} of oligonucleotides. For example, replacement of IVS2-654 5′ splice site with a consensus sequence, IVS2-654con, increased EC_{50} of oligonucleotides 12-fold, from 115 nM for ON-654 to 1,420 nM for ON-654con (Table 2). This is in spite of the fact that ON-654con has the highest GC content and *Tm* of all the oligonucleotides tested (Table 1) and therefore is expected to have the highest affinity to its target sequence. However, oligonucleotide sensitivity of the 5′ splice sites correlates better with the length of the aberrant, upstream exon (IVS2-654 $<$ -705 $<$ -745) than with the splice-site strength defined either as a match to the 5′ splice site consensus sequence (IVS2-705 $>$ -654 $>$ -745; data not shown, Senapathy et al., 1990) or in terms of the number of base-pairs formed with U1 snRNA (IVS2-705 $>$ -654 $=$ -745; Table 2). Since the variable part of the internal exons does not seem to contain typical splicing enhancers or silencers (Fig. 1B), it seems likely that it is the length of the aberrant exon that

modulates its recognition. The effects of exon length on splicing efficiency and exon inclusion both in vitro and in vivo have been recognized previously (Reed & Maniatis, 1986; Furdon & Kole, 1988; Dominski & Kole, 1991; Sterner & Berget, 1993). Nevertheless, the fact that aberrant exons IVS2-654 and IVS2-705, which in vivo are both fully included into the spliced product, differ in their respective sensitivity to the antisense oligonucleotides at the 5′ splice site by 29-fold was surprising. Note that a trace of correctly spliced product is detectable in untreated IVS2-705 but not in IVS2-654 HeLa cells (cf. lanes 1 in Figs. 3 and 2, respectively). Thus the efficiency of exon inclusion and its sensitivity to oligonucleotides are in qualitative if not quantitative agreement. A similar correlation was observed in vitro in cell-free extracts (Dominski & Kole, 1993, 1994a) Accordingly, the differences in sensitivity to the oligonucleotides are even more striking for IVS2-654 and -745 exons, the latter being partly included in the aberrant β-globin mRNA in the absence of the oligonucleotide (Fig. 4, lane 1 and Table 2).

The fact that consensus mutations at the 5′ splice sites in IVS2-654 and IVS2-705 mutants affect sensitivity of the 3′ splice site to ON-3′cr oligonucleotide are consistent with the concept of exon bridging (Kuo et al., 1991; Hertel et al., 1997) inherent in the exon definition model (Robberson et al., 1990). In this interpretation, the ability of ON-3′cr oligonucleotide to inhibit aberrant splicing of IVS2-705con but not of IVS2-654con pre-mRNA points to differences in bridging and recognition of the two exons flanked by the same 3′ splice site upstream and almost identical consensus 5′ splice site downstream (Table 2).

Whether the kinetic or steric hindrance models are responsible for the differences in the ability of the oligonucleotides to correct splicing of mutant pre-mRNAs, it appears that the oligonucleotides compete with the splicing factors for the target sequences and that the interactions of the latter with pre-mRNAs vary significantly. Although the experiments did not identify the competing factors, they did indicate that antisense oligonucleotides may provide useful tools for modifying and probing the interactions of the spliceosomes with the pre-mRNAs.

MATERIALS AND METHODS

Cells

Human β-globin genes carrying thalassemic mutations IVS2-654 and -705 were cloned under the CMV promoter as described (Dominski & Kole, 1994b). Additional mutants (IVS2-745, IVS2-654con, and IVS2-705 con) were obtained by PCR-based site-specific mutagenesis (Jones & Winistorfer, 1992). The details of the construction are available on request. The plasmids were cotransfected with a neomycin resistance plasmid in complex with Lipofectamine (Gibco-BRL) into HeLa cells and the cells stably expressing the mutated globin genes were isolated by G-418 antibiotic selection. The cells were grown in S-MEM supplemented with 5% fetal calf and 5% horse sera. For all experiments cells were plated in 24-well plates at 10^5 cells per well 24 h before treatment.

Oligonucleotide treatment

The 2′-*O*-methyl-oligoribonucleoside phosphorothioates (prepared and purified at Hybridon, Inc., Cambridge, Massachusetts) were used. The cells were treated with oligonucleotides complexed with Lipofectamine for 10 h and harvested 30 h later for subsequent analysis (see below). The sequences of the oligonucleotides are listed in Table 1. An oligonucleotide with a random sequence (ON-ran) was used as a control in the experiment illustrated in Figure 3A.

RNA analysis

Total RNA was isolated with TRI-Reagent (MRC, Cincinnati, Ohio) and 200 ng were analyzed by reverse transcription and PCR (RT-PCR) using rTth DNA polymerase as suggested by the manufacturer (Perkin-Elmer). The RT-PCR products were separated on 7.5% nondenaturing polyacrylamide gels. The RT-PCR was carried out with a-^{32}P-dATP for no more than 18–22 cycles. Under these conditions the amount of the PCR product was proportional to the amount of input RNA as were the relative amounts of PCR products generated from aberrantly and correctly spliced RNA (Chen & Chasin, 1993 and data not shown). No products were detectable without the reverse transcription step. For analysis of aberrantly and correctly spliced products, forward and reverse primers spanned, respectively, positions 21–43 of exon 2 (primer a, Fig. 1A) and positions 6–28 of exon 3 (primer c, Fig. 1A) of the human β-globin gene. For RT-PCR analysis of the expression of β-globin pre-mRNAs in HeLa cells, primer a was used in combination with primer b (Fig. 1A), spanning positions 119–143 of β-globin intron 2.

Protein analysis

Immediately preceding the isolation of protein, the cells were treated with hemin (10 μM, Fluka, Switzerland) in serum-free medium for 4 h. The cells were washed with HBSS and lysed for 10 min in 75 μL of 3% SDS, 60 mM Tris, pH 6.8, 7% sucrose, 1 mM EDTA, pH 7.4, 2 mg/mL aprotonin, 2 mg/mL leupeptin, PMSF 100 mg/mL. Blots of proteins separated on a 10% Tricine-SDS polyacrylamide gel (Schägger & von Jagov, 1987) were incubated with polyclonal affinity-purified chicken anti-human hemoglobin IgG as primary antibody and rabbit anti-chicken horseradish peroxidase-conjugated IgG as secondary antibody (Accurate Chemicals, Westbury, New York). Subsequently, the blots were developed with the ECL detection system (Amersham).

Image processing and quantitation of the data

Autoradiograms of RT-PCR gels were captured by DAGE MTI CCD72 video camera (Michigan City, Indiana) and the

images were processed using NIH Image 1.61 and MacDraw Pro 1.0 software. The final figures were printed on a Tektronix phaser 550 printer. The NIH Image 1.61 was also used for quantitation of the autoradiograms. The percent of the correct product relative to the sum of correct and aberrant products was calculated and linear regression analysis of the data was used to determine EC_{50} of the oligonucleotides. Note that the ratios of AMP content in the aberrant and correct products for IVS2-654, -705, and -745 mutants are 1.57, 2.02, and 2.31, respectively. They were factored in the calculations of correction of splicing and EC_{50} values.

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