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Inhibition of Splicing of Wild-Type and Mutated Luciferase-Adenovirus Pre-mRNAs by Antisense Oligonucleotides

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SUMMARY

We report the construction, characterization, and use of luciferase reporters to test the ability of antisense oligonucleotides to inhibit RNA splicing. β-Globin and adenovirus introns were inserted into a luciferase cDNA, and luciferase expression was analyzed in transiently transfected cells. The adenovirus reporter expressed large amounts of luciferase, but two β-globin constructs were inactive. RNA analyses determined that the β-globin pre-mRNAs were not spliced. Mutagenesis of the β-globin 5′ splice site, branchpoint, and 3′ splice site sequences to the adenovirus intron sequences promoted maximal splicing and luciferase activity; reciprocal changes in all three elements of the adenovirus intron eliminated luciferase activity. Wild-type and 3′ splice site mutated adenovirus reporters were used to determine the ability of phosphorothioate deoxy and 2′ methoxy oligonucleotides to inhibit splicing. RNase H activating oligodeoxynucleotides were better inhibitors of wild-type adenovirus expression than were 2′ methoxy analogues. However, 2′ methoxy oligonucleotides specific for the branchpoint were more effective inhibitors of splicing of adenovirus transcript containing the β-globin branchpoint and 3′ splice site. We suggest that pre-mRNAs with weak splice sites are potential targets for oligonucleotides that inhibit splicing by occupancy rather than cleavage of the transcripts.

During the past several years, substantial interest in the development of antisense oligonucleotides as therapeutic agents has evolved (for reviews, see Refs. 1–6). Although significant advances have been made in understanding the pharmacological properties of antisense oligonucleotides, a detailed understanding of the mechanism of action and factors that influence both the antisense and nonantisense activities of various classes of oligonucleotides is not yet developed. For example, a number of studies have shown that the potencies of phosphorothioate oligodeoxynucleotides vary widely, depending on the site in an RNA species to which they bind, even though they have nearly identical theoretical affinities for the RNA target sites and similar abilities to induce the degradation of the targeted RNAs by serving as substrates for RNase H (4; for a review, see Refs. 5 and 6).

One useful way of thinking about the mechanisms of action of antisense oligonucleotides is as inhibitors of the intermediary metabolism of target RNA species. After binding to target RNAs, antisense oligonucleotides may enhance the rate of degradation of the target RNA (e.g., activate RNase H) or disrupt the metabolic processes that result in effective utilization of the mature RNA (for reviews, see Refs. 1–6). In this context, it is clear that the rates of a number of events will influence the antisense potencies of oligonucleotides; these include overall transcriptional rate, the rate of processing of the pre-mRNA, the translational rate and rate of degradation of the RNA, as well as the various factors that determine the concentration of drug at the site of expression of the target RNA.

Most eukaryotic nuclear mRNAs are generated by the removal of noncoding introns from pre-mRNA through the process of splicing. Substantial progress has recently been made in understanding the basic mechanisms involved in this process (for reviews, see Refs. 7 and 8). Splicing occurs in two steps; the first cleavage step occurs by a nucleophilic attack of the 2′ hydroxyl of the BP adenosine on the phosphodiester bond at the 5′ SS that releases the splicing intermediates, the 5′ exon, and the intron lariat-3′ exon. In the second step, cleavage occurs at the 3′ SS with concomitant ligation of the two exons and release of the intron as a lariat. Conserved sequence elements at the 5′ SS, 3′ SS, and BP mediate these events through their interaction with a large number of snRNAs (U1, U2, U4/U6, and U5) and associated proteins, collectively known as snRNPs. Also, a large number of non-snRNP proteins are involved in the regulation of constitutive and alternative splicing.

ABBREVIATIONS: 3′ SS, 3′ splice site; 5′ SS, 5′ splice site; BP, branchpoint; FBS, fetal bovine serum; hglo, human β-globin; rglo, rabbit β-globin; snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; RT, reverse-transcriptase; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; 5LOH, intron H of human 5 lipoxygenase.

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TABLE 1
Sequence of Oligonucleotides Used to Construct Luciferase Reporter

Construct	Oligonucleotides
LI-12 Adeno WT 5′-WT 3′	(+) 5′GCTGAACGGTACCTCGAGGTGAGTACTCCCTCTCAAAAGC 3′
	(−) 5′GACTGCGAATTCCTCGAGAAAAAAAAAGGGACAGGATAAGT 3′
LI-12 hglo WT 5′-WT 3′	(+) 5′GCTGAACGAATTCCTCGAGGTTGGTATCAAGGTTACAAG 3′
	(−) 5′GACTGCGGTACCTCGAGGTGGGAAAATAGACCAATAGG 3′
LI-12 rglo WT 5′-WT 3′	(+) 5′GCTGAACGGTACCTCGAGGTTGGTATCCTTTTTACAGCAC 3′
	(−) 5′GACTGCGAATTCCTCGAGAAATGAAAACAGCCCAGGGGAG 3′
LI-12 5LOH WT 5′-WT 3′	(+) 5′GAACGAATTCCTCGAGGTACAGCCAGCTACCGCCCC 3′
	(−) 5′GACTGCGGTACCTCGAGAGGGGAGAGGAGGAGGCCTC 3′
LI-12 hglo (+) WT 5′-Py1	(+) 5′GCTGAACGAATTCCTCGAGGTTGGTATCAAGGTTACAAG 3′
	(−) 5′GGCGGCCTCGAGGAGAGAAAAGAGACCAATAGGCAGA 3′
LI-12 hglo (+) WT 5′-Py2	(+) 5′GCTGAACGAATTCCTCGAGGTTGGTATCAAGGTTACAAG 3′
	(−) 5′GGCGGCCTCGAGAAAAAAAAAGGAGACCAATAGGCAGAGAGAG 3′
LI-12 hglo (+) MT 5′-WT3′	(+) 5′GCTGAACGAATTCCTCGAGGTGAGTATCAAGGTTACAAGAC 3′
	(−) 5′GACTGCGGTACCTCGAGGTGGGAAAATAGACCAATAGG 3′
LI-12 hglo (+) MT 5′-Py1	(+) 5′GCTGAACGAATTCCTCGAGGTGAGTATCAAGGTTACAAGAC 3′
	(−) 5′GGCGGCCTCGAGGAGAGAAAAGAGACCAATAGGCAGA 3′
LI-12 hglo (+) MT 5′-Py2	(+) 5′GCTGAACGAATTCCTCGAGGTGAGTATCAAGGTTACAAGAC 3′
	(−) 5′GGCGGCCTCGAGAAAAAAAAAGGAGACCAATAGGCAGAGAGAG 3′
LI-12 hglo (+) WT 5′-BP-WT 3′	(+) 5′GCTGAACGAATTCCTCGAGGTTGGTATCAAGGTTACAAG 3′
	(−) 5′GCGGTACCTCGAGGTGGGAAAATAGACGAATAAGTAGAGAGAGTCAG 3′
LI-12 hglo (+) WT 5′-BP-Py2 3′	(+) 5′GCTGAACGAATTCCTCGAGGTTGGTATCAAGGTTACAAG 3′
	(−) 5′GGCGGCCTCGAGAAAAAAAAAGGAGACGAATAAGTAGAGAGAGTCAG 3′
LI-12 hglo (+) MT 5′-BP-WT 3′	(+) 5′GCTGAACGAATTCCTCGAGGTGAGTATCAAGGTTACAAGAC 3′
	(−) 5′GCGGTACCTCGAGGTGGGAAAATAGACGAATAAGTAGAGAGAGTCAG 3′
LI-12 hglo (+) MT 5′-BP-Py2 3′	(+) 5′GCTGAACGAATTCCTCGAGGTGAGTATCAAGGTTACAAGAC 3′
	(−) 5′GGCGGCCTCGAGAAAAAAAAAGGAGACGAATAAGTAGAGAGAGTCAG 3′
LI-12 Ad (+) WT 5′-hglo 3′	(+) 5′GCTGAACGGTACCTCGAGGTGAGTACTCCCTCTCAAAAGC 3′
	(−) 5′GGCGGCCTCGAGGTGGGAAAATAGACCAATAAGTATGACATCATCAAG 3′
LI-12 Ad (+) hglo 5′-WT 3′	(+) 5′CCGGCGGCCTCGAGGTTGGTACTCCCTCTCAAAAGC 3′
	(−) 5′GACTGCGAATTCCTCGAGAAAAAAAAAGGGACAGGATAAGT 3′
LI-12 Ad (+) hglo 5′-hglo 3′	(+) 5′CCGGCGGCCTCGAGGTTGGTACTCCCTCTCAAAAGC 3′
	(−) 5′GGCGGCCTCGAGGTGGGAAAATAGACCAATAAGTATGACATCATCAAG 3′

Although there are reports of inhibition of splicing by antisense oligonucleotides (for a review, see Ref. 9), efforts to systematically investigate the factors that might influence the abilities of antisense oligonucleotides to inhibit splicing have not been reported.

The class of antisense oligonucleotides that is best understood is phosphorothioate oligodeoxynucleotides (for reviews, see Refs. 1–6). Phosphorothioate oligodeoxynucleotides have displayed effective parenteral pharmacokinetics (for a review, see Ref. 9). Moreover, this class of oligonucleotides has displayed impressive pharmacological activities consistent with an antisense mechanism in a variety of animal models (10, 11; for a review, see Ref. 9). When phosphorothioate oligodeoxynucleotides bind to a target RNA, the resulting duplex serves as a substrate for the DNA/RNA duplex-specific nuclease, RNase H (12). Substitution in the 2′ position of the antisense oligonucleotide (e.g., 2′ methyl) results in a duplex that does not serve as an RNase H substrate (12, 13).

The hypothesis we tested is that optimal antisense splicing inhibitors can be rationally designed when the rate-limiting splicing steps are understood. The objectives of the present study were, therefore, to (a) develop a facile, relatively quantitative assay of splicing events in mammalian cells; (b) confirm that, with proper controls, an assay of the translation of a protein product, such as luciferase, can be used as a surrogate end point for splicing events; (c) determine if there is a hierarchy of sites within introns relative to their sensitivity to antisense inhibition of splicing; (d) determine if conserved intron sequences (5′ SS, 3′ SS, or polypyrimidine tract) are good targets for antisense drugs; and (e) determine if reasonable judgments regarding the rates with which introns are spliced (optimal antisense target introns) can be based simply on intron sequences.

To achieve these objectives, we constructed a luciferase reporter plasmid capable of accepting one or more intron cassettes. A variety of wild-type and mutant introns were then cloned into the plasmid and tested in a transient transfection assay to determine the influence of various RNA sequences on splicing. Once validated, the assay was used to evaluate the effects of putative antisense oligonucleotides designed to inhibit splicing. We compared the activities of phosphorothioate oligodeoxynucleotides with those of phosphorothioate oligonucleotides with 2′ methoxy substitutions throughout.

Materials and Methods

Construction of luciferase reporters. The parental plasmid for all luciferase constructions was LI-12, a luciferase cDNA that contains an insertion of an *Xho*1 site after amino acid residue 12 (14, 15). Wild-type and mutant introns were PCR amplified (16) using plasmid templates containing adenovirus 2 major late transcription unit intron 1,[1] hglo intron 1,[2] rglo intron 1,[3] or 5LOH[4] and the oligonucleotides shown in Table 1. LI-12 and the PCR products were digested with *Xho*1, gel purified using Gene Clean and Mermaid Kits (BIO 101, Vista, CA), ligated, and transformed into DH5 α competent

[1] Sequence shown in Padgett *et al.* (35); plasmid obtained from E. Sanheimer, Yale University.

[2] Sequence shown in Lawn *et al.* (36); plasmid obtained from R. Reed, Harvard University.

[3] Sequence given in Van Ooyen *et al.* (37); plasmid obtained from B. Monia, Isis Pharmaceuticals.

[4] Plasmid obtained from B. Monia, Isis Pharmaceuticals.

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cells (GIBCO-BRL, Gaithersburg, MD) using standard techniques (17). Mini-preps of DNA were obtained from potential positive clones using 1.5 ml of bacterial cultures and Qiagen spin columns (Qiagen, Chatsworth, CA). Mini-prep DNA was subjected to restriction enzyme digestion (17) and sequencing (Sequenase Kit; United States Biochemical Corp., Cleveland, OH) to identify constructs containing the introns in the desired orientation and to confirm the ligation junctions. The following oligonucleotides, which anneal to the 5′ or 3′ side of the LI-12 insertion site, were used for sequencing: upstream, 5′GAATGTCGCTCGCAGTGAC3′; and downstream, 5′CGTGATGTTCACCTCGATATGTGC3′. Highly purified DNA was prepared from 500-ml to 1-liter cultures of transformed bacteria using kits (Qiagen, Chatsworth, CA).

The intron sequence amplified by PCR begins with the first nucleotide at the 5′ end of the intron and ends at position -6 relative to the 3′ SS. This design ensures that if the luciferase pre-mRNA is spliced after the AG in the *Xho*1 site at the 3′ end, the *Xho*1 duplication is eliminated, and the original LI-12 sequence is restored. Because no additional amino acids are generated, correct removal of the intron should result in luciferase activity equivalent to the LI-12 cDNA. If the intron is not removed, stop codons present in the intron should eliminate luciferase activity.

Cell culture, transfections, and oligonucleotide treatment. HeLa cells were obtained from American Type Culture Collection (Rockville, MD) and propagated in low-glucose DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FBS. At 24–48 hr before transfection, cells were plated at a density of 1–3 × 10^5 cells/well in six-well plates (Falcon 3846). At this plating density, cells were 75–90% confluent at the time of transfection or oligonucleotide treatment. The calcium phosphate precipitation method (18) was used for transient transfections with the following modification; cells were treated with 2.5 μg/ml of lipofectin in serum-free OptiMEM (GIBCO-BRL) for 2 hr, followed by a 1–2-hr incubation in 1 ml of DMEM plus 10% FBS, before transfection. This modification eliminated the need for glycerol shock of the cells as lipofectin was more effective than glycerol in increasing the transfection efficiency. Cells were incubated with the calcium phosphate/DNA precipitate (0.1 ml containing 5 μg of test DNA and 5 μg of carrier DNA) for 8–12 hr. After being washed twice with PBS, cells were incubated in 2 ml of DMEM plus 10% FBS for the desired time before harvesting for luciferase assays or RNA preparations. For oligonucleotide treatments, 20 μM stocks of the oligonucleotides were heated at 68° for 10 min and quick cooled on ice, and the desired amount of oligonucleotides was added to tubes containing Opti-MEM plus lipofectin. One milliliter of the oligonucleotide/lipofectin/Opti-MEM mixture was added to the cells after they were washed twice with PBS to remove serum. For most experiments, cells were incubated with the oligonucleotides for 2 hr, washed once with PBS, and incubated 1–2 hr with DMEM plus 10% FBS before transfection. When dose-response curves were performed, the oligonucleotide/lipofectin ratio was maintained at 100 nM oligonucleotide/1.0 μg/ml lipofectin, but for oligonucleotide screening, a ratio of 150 nM oligonucleotide/2.5 μg/ml of lipofectin was used.

We studied the effects of transfection of increasing amounts of DNA using both the adenovirus/wild-type and cDNA constructs. Each construct was transfected at 1.25–10 μg DNA/well. In this range, the luciferase activity increased proportionally to increasing DNA dose. To limit variability in transfection efficiency, all transfections were performed using 5 μg of test DNA and 5 μg of carrier DNA per well in all experiments shown.

To further ensure that transfection efficiency was consistent and contributed no systematic errors that might confound interpretation, before the experiments were performed, transfection was optimized for cell number and protein content. Then, for each experiment, a sufficient number of plates of cells were prepared to allow selection of only those plates with optimal cell densities for experimentation. Furthermore, each point in the experiment was performed in triplicate, and all results shown represent the mean of six or more independent determinations. Also, each member of each triplicate was randomly assigned to separate plates to ensure that any plate-to-plate variation contributed to the standard errors shown in a fashion that could not result in misinterpretation of results. The standard errors observed (e.g., see Figs. 4A and 6) were quite small. Finally, all comparisons shown derive from experiments in which all samples were compared head-to-head on the same day in the same experiment.

Luciferase assays. Luciferase assays were performed in triplicate according to the basic protocol described in Ausubel *et al.* (18). After transfection for the desired times, HeLa cells were washed twice in PBS and placed on ice. Lysis buffer (200 μl/well) was added, and the cells were scraped loose from the plate and transferred to microcentrifuge tubes on ice. Typically, 50 μl of each lysate was assayed for luciferase activity immediately after harvest or after storage at −80° for no more than 2 days. A luminometer (ML1000, model 2.4, Dynatech Laboratories, Chantilly, VA) was used to quantify the light emitted on injection of luciferin into 96-well plates containing the cell lysates and other reaction components.

RNA preparations and RT-PCR analyses. After DNA transfection and/or oligonucleotide treatment, cells were washed twice with PBS, and RNA was prepared using RNazol B (Tel-Test, Friendswood, TX) according to the procedure recommended by the manufacturer. To ensure that there was no contaminating DNA in the RNA, the samples were treated with RQ1 RNase-free DNase I (Promega, Madison, WI), and the DNase was removed by Proteinase K treatment, two phenol/chloroform extractions, one chloroform extraction, and ethanol precipitation (17).

RT was performed for 1 hr at 37° in a 50-μl reaction volume containing 2.5 μg of each RNA preparation, 50 pmol of each of the reverse primers complementary to LI-12 exon 2 (5′CGTGATGTTCACCTCGATATGTGC3′) or endogenous G3PDH exon 4 (5′ AGATGGTGATGGGATTTCCA 3′), 10 μl of 5× Moloney murine leukemia virus RT buffer (1 × 15mM Tris·HCl, 75 mM KCl, 3 mM Mg Cl_2, pH 8.3) (GIBCO-BRL), 5 μl of 0.1 M DTT, 1 μl of Inhibit-ACE (5′ 3′, Boulder, CO), 2.5 μl of a 10 mM mix of deoxynucleotides (dGTP, dATP, dTTP, and dCTP) and 1 μl of Moloney murine leukemia virus RT (GIBCO-BRL). After heat inactivation at 95° for 5 min, 5 μl of the reaction was used in PCR reactions.

The forward primers used for the PCR reactions, LI-12 exon 1 (5′GAATGTCGCTCGCAGTGAC 3′) and G3PDH exon 3 (5′TATTGGGCGCCTGGTCACCA 3′), were 5′ end-labeled and gel purified using standard procedures (17). A typical 100-μl PCR reaction contained 10 μl of 10× *Taq* polymerase buffer (1 × 50 mM KCl, 10 mM Tris·HCl, pH 9.0 at 25°, 0.1% Triton X-100) (Promega, Madison, WI), 6 μl of 25 mM $MgCl_2$, 2 μl of a 10-mM mix of dNTPs, 1.0 μl each of the ^{32}P-labeled luciferase exon 1 and G3PDH exon 3 forward primers (0.2 pmol containing 0.5–1.5 × 10^6 cpm each), 1 μl (~5 pmol) of each of the unlabeled forward primers, 50 pmol each of the luciferase exon 2 and G3PDH exon 4 reverse primers (shown above), 1 μl of *Taq* polymerase (5 units/μl; Promega), and 5 μl of the RT products. PCR was performed for 1 cycle at 94° for 2 min, 15–30 cycles of 1 min at 94°, 1 min at 55°, and 1.5 min at 72°, and a final cycle of 2 min at 72° on an EriComp EZ Cycler (ERICOMP, San Diego, CA). Then, 1–5% of the PCR products were electrophoresed on 5% acrylamide 8 M urea gels that were exposed to film (Kodax XAR5) for 1 hr to overnight. The identity of each DNA species was determined by size comparison to 3′ end-labeled DNA fragments of known molecular weights derived from *Msp*1-digested pBR 322. The relative amounts of radioactivity in each gel band was determined by phosphorimager analysis using Image-Quant Version 3.0 (Molecular Dynamics, Sunnyvale, CA).

Preparation of antisense oligonucleotides. Synthesis of phosphorothioate (deoxy and 2′ modified) and phosphodiester oligonucleotides were performed using an Applied Biosystems 380B automated DNA synthesizer as previously described (19, 20). Purification of oligonucleotide products was also as previously described (19, 20). Purified oligonucleotide products were >90% full length and >98%

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N-2 or longer as determined by polyacrylamide gel electrophoresis. RNA substrates were further purified by polyacrylamide gel electrophoresis.

Results

Construction of luciferase splicing reporters. To assess the ability of antisense oligonucleotides to inhibit the splicing process, it was necessary to construct a reporter with the following characteristics: it should be cell based to be predictive of the *in vivo* efficacy of oligonucleotide treatment; measurement of the reporter output should be highly sensitive and reproducible over a wide range; and the assay should be rapid enough to screen a large number of oligonucleotides. Transient expression of a luciferase splicing reporter meets all of these criteria. We therefore inserted a number of introns into the unique *Xho*I site at amino acid position 12 of the firefly luciferase expression vector LI-12-ASRP (14, 15). The *Xho*I site had been inserted at this position by linker scanning mutagenesis and was previously shown to have no effect on correct localization of the enzyme, but the luciferase activity had not been determined. Transient transfection of LI-12 into HeLa cells confirmed that high luciferase activity was maintained even though four additional amino acids had been inserted at amino acid position 12 (data not shown). This vector had the additional advantage of a 3′ SS AG provided by the CTCGAG *Xho*I site. This allowed us to insert introns of interest that, when spliced properly, would maintain the luciferase reading frame and not add additional amino acids (Fig. 1A).

To analyze the kinetics of splicing, we wanted to obtain a variety of reporters that would splice with different efficiencies. We therefore PCR-amplified two β-globin introns, rglo and hglo, an adenovirus intron in the sense orientation [Ad(+)], an adenovirus intron in the antisense orientation [Ad(−)], and 5LOH intron in the sense orientation; these introns were then inserted into the *Xho*I site of LI-12 (Fig. 1B). Both β-globin introns and the sense adenovirus intron have been used previously for *in vitro* and cellular splicing studies and shown to splice efficiently (for reviews, see Refs. 7 and 8). The antisense adenovirus intron serves as a negative control, and the 5LOH intron reporter was constructed to analyze splicing of a longer intron.

Analysis of splicing of wild-type introns. Fig. 2A shows the results of a luciferase assay performed on lysates from HeLa cells transiently expressing the LI-12 cDNA or splicing reporters. Although the cDNA and the Ad(+) reporters expressed high levels of luciferase, the 5LOH and hglo constructs failed to express luciferase activity above the negative control [Ad(−)] levels. The rglo reporter expression was barely above background levels (note the log scale). Interestingly, we consistently observed that luciferase expression was enhanced 5–10-fold by the presence of the adenovirus intron [compare cDNA and Ad(+) in Fig. 2A].

The lack of luciferase expression from the 5LOH construct is not surprising since the 5LOH 5′ SS deviates significantly from the consensus sequence (Fig. 1B). However, the failure of the β-globin intron-containing vectors to express luciferase was surprising because these introns, as stated above, have been used extensively for *in vitro* splicing studies. We therefore characterized the expression of luciferase after transfection of each of the vectors as a function of time. Fig. 2B shows that luciferase expression deriving from the Ad(+) vector peaked between 20 and 24 hr after transfection. All of the vectors that resulted in luciferase expression displayed similar time courses. In contrast, no luciferase activity, in excess to that observed with the Ad(−) control, was detected at any time when the two globin intron-containing vectors were transfected (data not shown).

To determine if the results of the luciferase assay correspond to changes in the efficiency of splicing, the RNA generated from each of the luciferase reporter constructs was assayed using an RT-PCR approach shown in Fig. 3A. Fig. 3B confirms that significant amounts of luciferase mRNA were generated only from luciferase cDNA and Ad(+) constructs, whereas the β-globin constructs were transcribed but not spliced (note the accumulation of pre-mRNA). Although the β-globin and Ad(−) pre-mRNAs were quite stable, we were unable to detect 5LOH pre-mRNA.

Optimal 5′ and 3′ SS and BP are required for maximal splicing. The sequences of the 5′ SS, BP, and polypyrimidine tract downstream of the BP of the adenovirus intron are a better match to all of the consensus sequences than the sequences in the β-globin introns (Fig. 1B). In addition, the Ad(+) BP is located nearer to the 3′ SS than either of the β-globin BPs. We therefore altered these elements of the hglo intron so that they more closely resembled those of the Ad(+) intron to determine the contribution of each to the splicing efficiency (Fig. 1C). Complementary changes in the splice sites were also made in the adenovirus intron (Fig. 1D).

Improvement of the hglo polypyrimidine tract (Fig. 4A, *lanes 4 and 5*) or 5′ SS (Fig. 4A, *lane 6*) increased luciferase expression, but it was still very near the background levels determined by Ad(−) expression (Fig. 4A, *lane 10*). Luciferase activity was increased significantly above background when the hglo BP was changed (both sequence and location) to that of the adenovirus sequence (Fig. 4A, *lane 11*), but the activity was still several orders of magnitude less than wild-type Ad(+) (Fig. 4A, *lane 9*). Combinations of changes in two of the three conserved sequence elements increased the luciferase activity even further (Fig. 4A, *lanes 7, 8, 12, and 13*) but to approximately the same extent for each combination. Luciferase activity was increased to levels equal to those of wild-type Ad(+) by conversion of all three β-globin splicing elements to that of Ad(+) (Fig. 4A, compare *lanes 9 and 14*). Likewise, mutations in the adenovirus intron that changed either the 5′ SS (Fig. 4A, *lane 16*) or the 3′ SS (BP and polypyrimidine tract; Fig. 4A, *lane 15*) to the globin sequences decreased, but did not eliminate, luciferase activity, whereas alteration of both sites decreased luciferase activity to background levels (Fig. 4A, *lane 17*).

The luciferase results from the hybrid constructs were confirmed by RT-PCR analysis of RNA purified from transfected HeLa cells (Fig. 4B). Large amounts of luciferase mRNA were generated only when at least two of the splicing elements were optimal, and maximal mRNA product occurred when all three matched the consensus sequences. Pre-mRNA levels were inversely proportional to mRNA levels and accumulated to highest levels when the splicing signals were weak (Fig. 4C). A comparison of mRNA levels with luciferase activity derived from each of the splicing constructs is shown in Fig. 4D. Clearly, mRNA levels and luciferase activity correlated well, validating the use of the luciferase assay as a measure of splicing efficiency.

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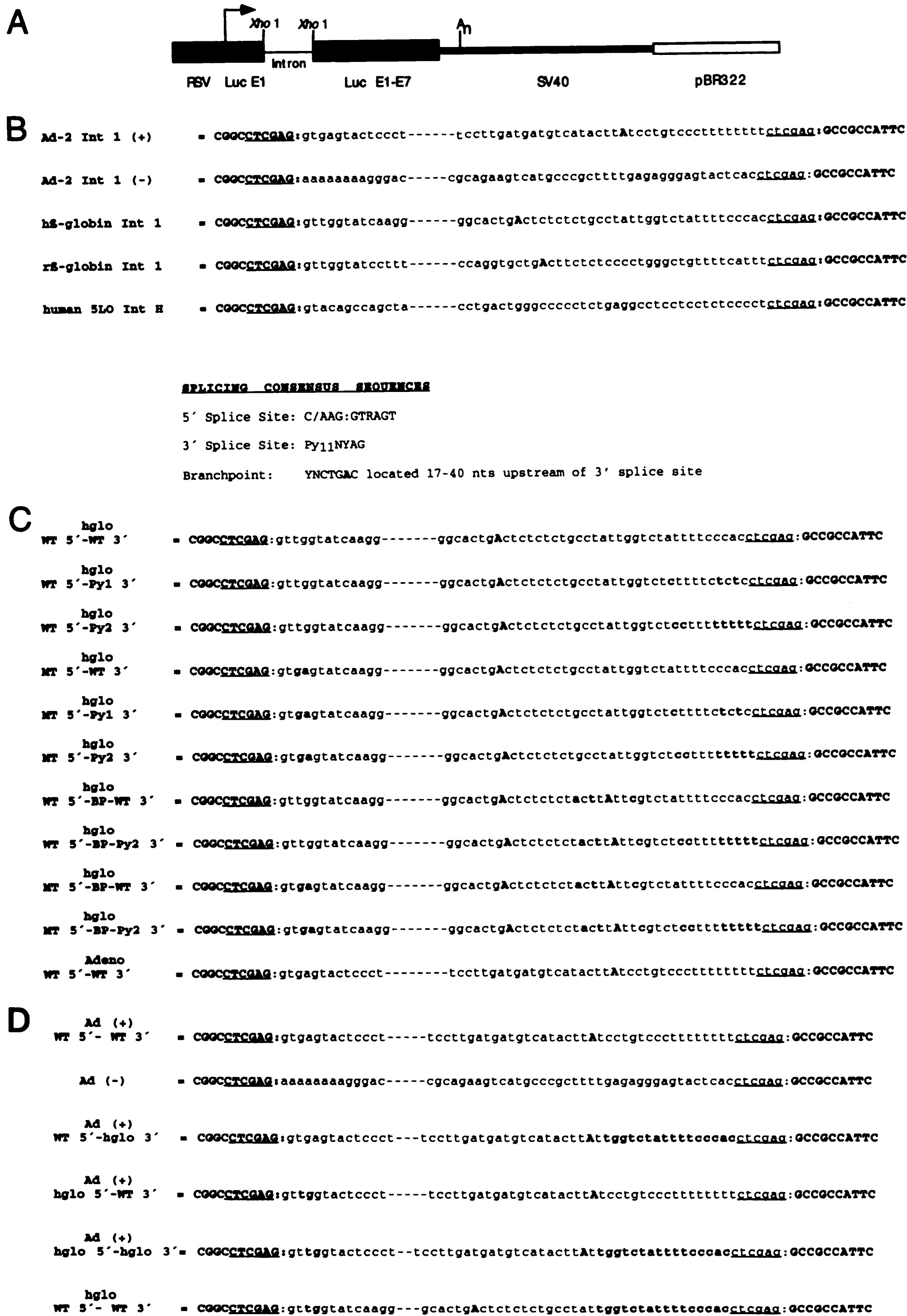

Fig. 1. A, Structure of the luciferase (*Luc*) expression vector LI-12. LI-12 is a derivative of pRSV-L containing an insertion of an *Xho*I restriction enzyme site at amino acid position 12 that is described in De Wet *et al.* (14). *Striped area*, RSV promoter; *shaded area*, luciferase cDNA; *thin line*, intron; *arrow*, location and direction of transcription initiation; *solid bar*, SV40 sequences containing the small t-antigen intron and polyadenylation signal (A_n); *open bar*, pBR322 region required for propagation in bacteria. (Diagram is not drawn to scale.) B, Nucleotide sequences of the splice junctions of wild-type introns inserted into LI-12. *Bold uppercase letters*, LI-12 coding regions flanking the site of intron insertion. *Lowercase letters*, inserted intron sequences. *Underlined sequence*, *Xho*1 site. *Dashes*, intron sequences between the 5′ SS and 3′ SS that are not shown. Consensus sequences for the 5′ SS and 3′ SS and BP are indicated with the known adenosine BP shown in *bold uppercase*. *r*, purine; *Py*, polypyrimidine; *Y*, pyrimidinel; *N*, any nucleotide. C, Comparison of the nucleotide sequences of the splice junctions of wild-type and mutant derivatives of LI-12 hglo with LI-12 adenovirus splicing reporters. Nucleotides that differ from the wild-type sequence are shown in *bold type*. *R*, purine; *Py*, polypyrimidine; *Y*, pyrimidine; *N*, any nucleotide. D, Comparison of the nucleotide sequence of the splice junctions of wild-type and mutant derivatives of LI-12 adenovirus with LI-12 hglo splicing reporters.

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Identification of antisense oligonucleotides that inhibit splicing. Having developed and validated an assay that supports testing of the activities of putative antisense inhibitors, we wanted to determine if the potency of antisense inhibition of splicing was dependent on splice site strength and splicing efficiency. We tested the ability of antisense oligonucleotides to inhibit splicing of two of the luciferase transcripts, which were shown in the above studies to differ greatly in their splicing efficiencies. LI-12 Ad(+) and LI-12 Ad wild-type 5′-hglo 3′ were chosen because the levels of spliced products generated from them differed by 3–4-fold, but luciferase activity from both was large enough to detect decreases in activity on antisense treatment. Fig. 5, A and B, show the nucleotide sequences of these two transcripts and the regions targeted by antisense oligonucleotides. The primary targets were the 5′ and 3′ SS, but we also randomly chose four sites between the 5′ SS and BP. A site upstream of the 5′ SS was also chosen because of the presence of a

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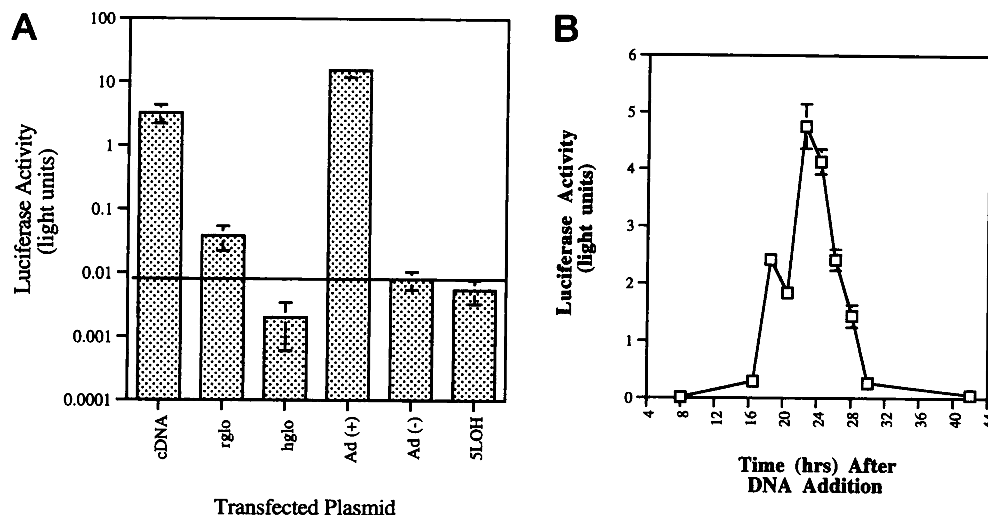

Fig. 2. A, Comparison of luciferase expression from LI-12 splicing reporters. Luciferase activity of extracts prepared from HeLa cells after transient transfection with the LI-12 reporter containing no intron (*cDNA*), rglo intron 1, hglo intron 1, adenovirus intron 1 in the sense orientation [*Ad*(+)] or the antisense orientation [*Ad*(−)], or 5LO. *All values below the horizontal line* are considered to be negative based on luciferase activity obtained from the negative control [Ad(−)]. B, Time course of LI-12 adenovirus expression. Luciferase assays were performed on extracts prepared from HeLa cells at the times shown after transfection (DNA addition) with the LI-12 adenovirus wild-type splicing reporter.

potential 5′ SS. Table 2 lists the antisense oligonucleotides used in the present study.

Fig. 6 compares the data from experiments in which HeLa cells were treated with antisense oligonucleotides and then transiently transfected with either LI-12 Ad wild-type or LI-12 Ad wild-type 5′-hglo 3′ splicing reporters. For both experiments, cells were treated with oligonucleotides at a concentration of 150 nM in the presence of 2.5 μg/ml of lipofectin for 2 hr, washed, and allowed to recover for 2 hr before transfection. Data from antisense treatment of the wild-type luciferase reporter LI-12 Ad shows that the phosphorothioate oligodeoxynucleotides were more effective than the 2′ methoxy oligonucleotides at decreasing luciferase expression (compare Fig. 6, A and B).

The 5′ SS was relatively resistant to inhibition by either class of oligonucleotide (Fig. 6). This implies that this region is inaccessible to the oligonucleotides or that the affinity of splicing factors for this region is much greater than that of the antisense oligonucleotides.

The effects of antisense oligonucleotides targeting the region between the 5′ SS and the BP were different for the two luciferase splicing reporters. Although oligodeoxynucleotides (8532–8535) were quite effective at inhibiting luciferase expression from the LI-12 Ad(+) reporter, the 2′ methoxy oligonucleotides (8543–8546) inhibited expression from this reporter only slightly. Antisense treatment of cells expressing the LI-12 Ad wild-type 5′-hglo 3′ reporter with the oligodeoxynucleotides (8532–8535) inhibited luciferase expression to approximately the same extent as observed with the wild-type reporter (Fig. 6, A and C). However, the 2′ methoxy oligonucleotides (8543–8546) actually increased luciferase expression from the LI-12 Ad wild-type 5′-hglo 3′ reporter (Fig. 6D). Because this reporter contains the same target sequences for 8543–8546 as the wild-type reporter, the alterations at the BP and 3′ SS must mediate this activation, possibly by altering RNA structure.

Data from antisense inhibition of splicing at the 3′ SS are more difficult to interpret. Analysis of the results of oligonucleotide treatment of wild-type LI-12 Ad indicates that the oligodeoxynucleotides were slightly more inhibitory than the 2′ methoxy oligonucleotides (compare 8536–8539 with 8547–8550 in Fig. 6, A and B). Because oligodeoxynucleotide/RNA duplexes can serve as substrates for RNase H, cleavage of the wild-type adenovirus pre-mRNA by RNase H may contribute to the enhanced efficacy of the oligodeoxynucleotides. A similar comparison of results from antisense treatment of LI-12 Ad wild-type 5′-hglo 3′ transfected cells shows the reverse relationship; the 2′ methoxy oligonucleotides were more potent inhibitors of luciferase expression than were the oligodeoxynucleotides (compare 8536 GLO-8539 GLO with 8547 GLO-8550 GLO in Fig. 6, C and D). The differences between the two classes of oligonucleotides is especially noticeable for oligonucleotides centered over the BP, where luciferase activity was decreased from ~50% (8537 GLO) to ~22% (8548 GLO) of control values. A possible explanation for such differences is that the higher affinity of the 2′ methoxy oligonucleotides (relative to the oligodeoxynucleotides) allows them to compete more effectively with splicing factors for binding to the weakened BP and 3′ SS present in this pre-mRNA.

Specificity of antisense oligonucleotide inhibition of splicing. The specificity of inhibition of LI-12 Ad wild-type 5′-hglo 3′ expression by 8548 GLO was demonstrated in comparison with the effect of 8548 on expression of this luciferase reporter. Both oligonucleotides are centered over the BP, but although 8548 GLO is completely complementary to the 3′ mutant pre-mRNA, 8548 is mismatched at 8 of 22 nucleotides just 3′ of the BP and therefore should bind with lower affinity. Fig. 6 shows that 8548 and 8548 GLO decreased luciferase expression from this reporter to ~79% and 27% of control values, respectively, suggesting that the observed decrease in luciferase activity is mediated by oligonucleotide binding to RNA. The specificity of 8548 GLO action was further confirmed by the dose-response curve shown in Fig. 7. At a concentration of ~125 nM, 8548 GLO inhibited luciferase expression from LI-12 Ad wild-type 5′-hglo 3′ to 50% of control levels, whereas it inhibited expression from wild-type LI-12 Ad by only ~10%. At higher concentrations,

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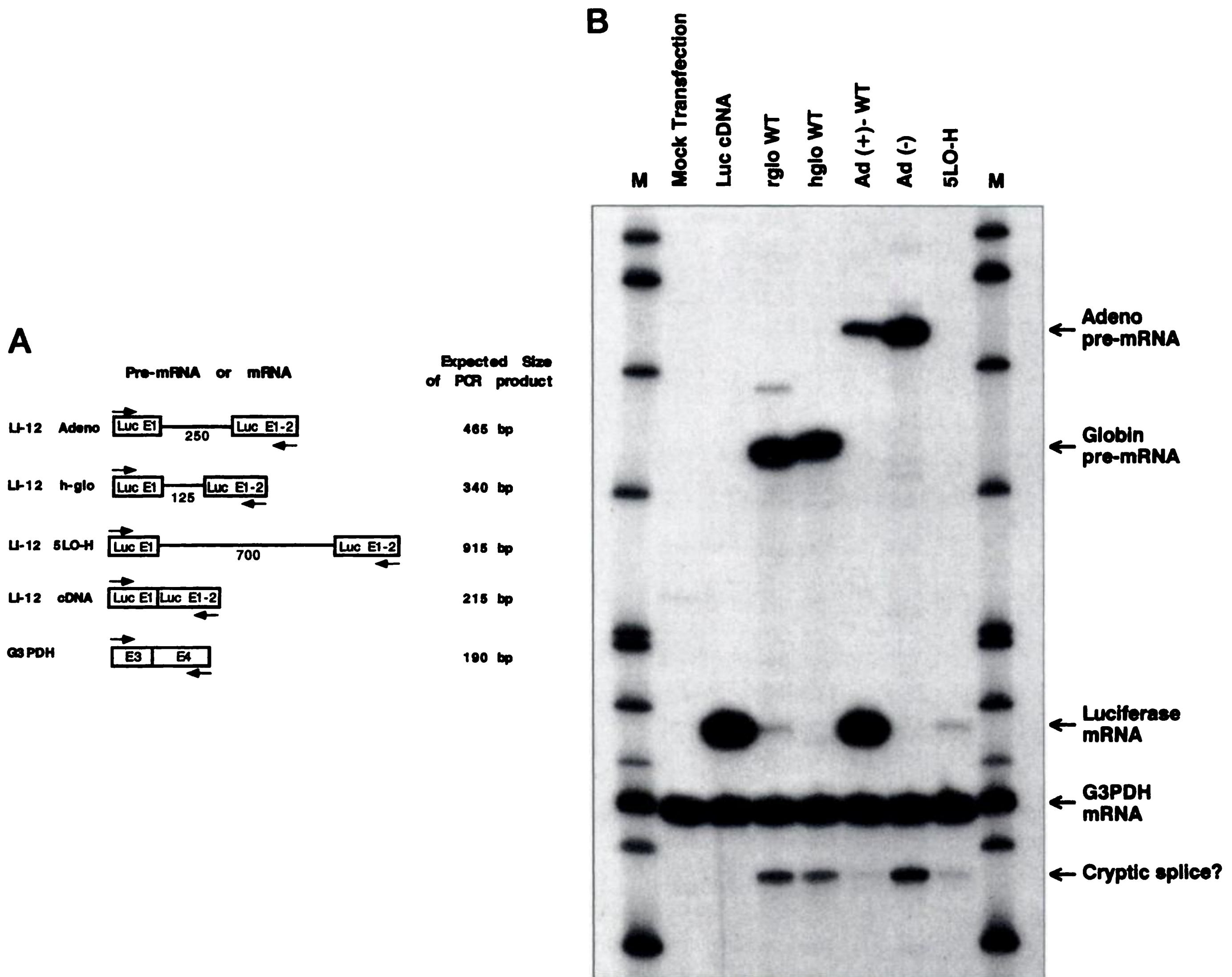

Fig. 3. A, Schematic of RT-PCR templates and expected products. The exon-intron structure of the pre-mRNA and mRNA in the region surrounding the intron insertions into LI-12 is shown with exons (*boxes*) and introns (*lines*). The region of the endogenous G3PDH mRNA that was amplified as a positive control is also shown. *Arrows* indicate the location of forward and reverse primers used for RT-PCR. The size of the introns and expected PCR products are shown in basepairs (bp). B, RT-PCR analysis of RNA from HeLa cells transiently transfected with wild-type splicing reporters. ^{32}P-5′-end-labeled forward primers corresponding to luciferase exon 1 and G3PDH exon 3, and unlabeled primers complementary to luciferase exon 2 or G3PDH exon 4 were used for RT-/PCR amplication (30 cycles) of total RNA prepared from HeLa cells transfected with the luciferase reporters shown. Amplified products were analyzed on a 5% acrylamide 8 M urea gel, and autoradiography was performed (4 hr, −80° exposure to XAR 5 film with an intensifying screen); the identity of the pre-mRNA and mRNA products is based on size determined by comparison to ^{32}P-3′-end-labeled pBR322 *Msp*1 fragments of known size (*M*).

8548 GLO and other phosphorothioates displayed non-sequence-specific inhibition of splicing (data not shown).

A comparison of base composition of active versus inactive oligonucleotides shows no correlation of inhibitory effects with base composition. The fact that many oligonucleotides tested were inactive also shows that the effects were not simply due to nonspecific phosphorothioate activities. Finally, in other experiments (data not shown), we have shown that increasing the length of an active oligonucleotide (ISIS 8548) increases potency as would be expected if the activity were due to hybridization to target RNA sequences.

Oligonucleotide inhibition of luciferase activity is due to inhibition of splicing. To confirm that effects described above were due to inhibition of splicing, we performed RT-PCR analysis of RNA purified from transfected cells after treatment with selected oligonucleotides. The RNA analyzed in Fig. 8 was prepared from transfections parallel to those shown in Fig. 6, C and D. Comparison of luciferase mRNA from 8544- and 8546-treated cells to the untreated control (lipofectin treated but no oligonucleotide) cells clearly shows that the mRNA levels were increased by treatment with these oligonucleotides (Fig. 8A). In addition, the pre-mRNA was decreased in these samples. Opposite changes were observed with 8548 GLO and 8549 GLO treatments, where the luciferase mRNA was decreased and the pre-mRNA was increased relative to the untreated and 8548-treated controls. Phosphorimager analysis of the gel shown in Fig. 8A (RT-PCR analysis performed with 25 cycles to ensure linearity) and normalization of the luciferase mRNA and pre-mRNA values to G3PDH levels confirmed the inverse relationships

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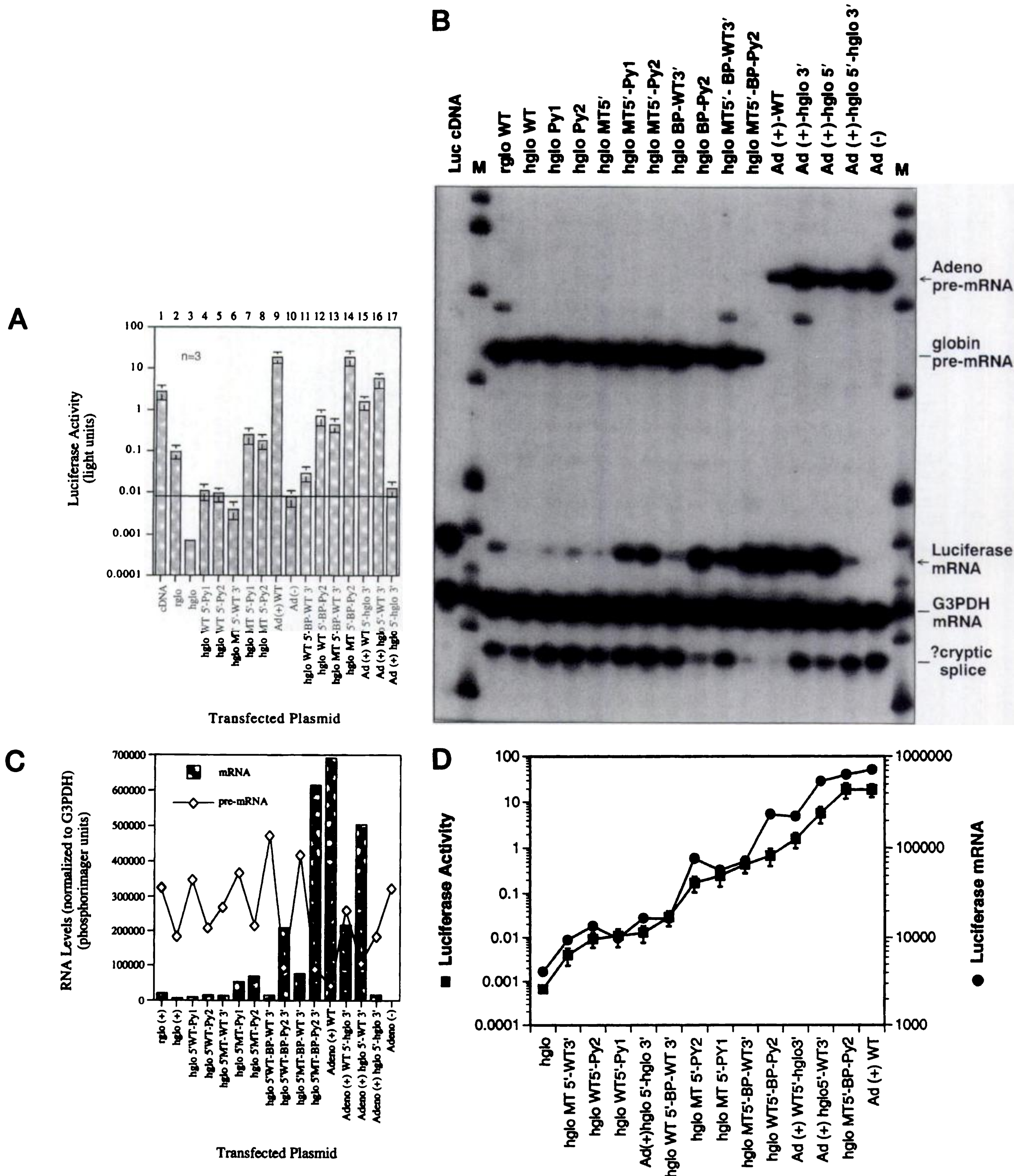

Fig. 4. A, Luciferase expression from LI-12 reporters containing wild-type or mutant adenovirus or β-globin introns. Luciferase assays were performed on extracts of HeLa cells transiently transfected with LI-12 splicing reporters containing wild-type or mutant adenovirus and hglo introns. The sequences at the 5′ SS, 3′ SS, and BP for each construct are shown in Fig. 1, C and D. B, RT-PCR analysis of RNA from HeLa cells transiently transfected with LI-12 reporters containing wild-type or mutant adenovirus or β-globin introns. RT-PCR and gel analysis were performed as described in the legend for Fig. 3B. Autoradiography was performed for 5 hr at −80° with an intensifying screen. C, Quantitative comparison of luciferase mRNA and pre-mRNA levels from wild-type and mutant luciferase reporters. The amount of radioactivity in the luciferase mRNA, luciferase pre-mRNA, and G3PDH mRNA bands on the gel shown in Fig. 4B was quantified by phosphorimager analyses. The luciferase pre-mRNA and mRNA values were normalized to the G3PDH levels and plotted. D, Comparison of luciferase activity and luciferase mRNA levels for adenovirus and hglo wild-type and mutant transfectants. Data from Fig. 4, A through C, were compared by ordering the data from lowest to highest luciferase activities and plotting the luciferase activities and mRNA levels on the same graph.

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TABLE 2
Sequence of Antisense Oligonucleotides

Sequence	Deoxy	2′ OMe
5′ CCATTTTACCAACAGTACCG 3′	ISIS 8529	ISIS 8540
5′ GAGTACTCACCTCGAGGCCG 3′	ISIS 8530	ISIS 8541
5′ TGAGAGGGAGTACTCACCTC 3′	ISIS 8531	ISIS 8542
5′ CTTAGCGCAGAAGTCATGCC 3′	ISIS 8532	ISIS 8543
5′ AGGCATCACCGCGGGCCAGG 3′	ISIS 8533	ISIS 8544
5′ AGATGGACGCGGCCACCCTC 3′	ISIS 8534	ISIS 8545
5′ CTACTGCGCCCTAGACGTGC 3′	ISIS 8535	ISIS 8546
5′ GGATAAGTATGACATCATCAAGG 3′	ISIS 8536	ISIS 8547
5′ CAATAAGTATGACATCATCAAGG 3′	ISIS 8536 Glo (10315)	ISIS 8547 Glo (10316)
5′ AAGGGACAGGATAAGTATGACA 3′	ISIS 8537	ISIS 8548
5′ AAATAGACCAATAAGTATGACA 3′	ISIS 8537 Glo (10317)	ISIS 8548 Glo (10318)
5′ GAAAAAAAAGGGACAGGATAAGT 3′	ISIS 8538	ISIS 8549
5′ GGTGGGAAAATAGACCAATAAGT 3′	ISIS 8538 Glo (10319)	ISIS 8549 Glo (10320)
5′ GCGGCCTCGAGAAAAAAAAGGGA 3′	ISIS 8539	ISIS 8550
5′ GCGGCCTCGAGGTGGGAAAATAG 3′	ISIS 8539 Glo (10321)	ISIS 8550 Glo (10322)

between mRNA and pre-mRNA levels (Fig. 8B). The RNA analysis confirms the luciferase data shown in Fig. 6D and demonstrates that inhibition of splicing can be analyzed using the simpler luciferase assay.

Discussion

Antisense oligonucleotides have been shown to be effective inhibitors of splicing *in vitro* when targeted to snRNAs (21–24) or to introns (25, 26). Inhibition of gene expression by antisense oligonucleotides targeting introns (27) and splice sites (28) in cultured cells has also been reported, but these studies did not show direct effects on splicing. A rational approach to the design of antisense drugs that inhibit splicing requires a better understanding of the factors that influence the rates of splicing *in vivo*. The splicing reporters we have analyzed suggest that reasonable judgments regarding selection of pre-mRNA targets for antisense inhibition *in vivo* can be made based on sequences at the 5′ SS, BP, and 3′ SS of the introns.

Our assay system used transient transfection of luciferase reporters whose expression was dependent on proper splicing. Because introns were inserted within the luciferase coding region, inefficient or incorrect splicing would result in decreased luciferase activity due to translational frame shifts, incorporation of additional amino acids, and/or premature termination of translation. The site of intron insertion into the luciferase coding region was chosen so that additional amino acids were not included if proper splicing occurred. This was critical because insertion of additional amino acids would make it difficult to determine if observed differences in luciferase activity were due to the altered protein or to differences in splicing. The luciferase reporters were further characterized by RNA analyses to confirm that the effects we observed with the mutations or antisense oligonucleotides in luciferase protein assays were due to modulation of splicing events. The luciferase pre-mRNA and mRNA levels correlated well with the luciferase enzyme activity and confirmed that the simpler and more rapid luciferase assays can be used to analyze splicing efficiencies or antisense inhibition of splicing.

Since the initial development of *in vitro* splicing systems, the small β-globin introns have been used extensively to study splicing (for reviews, see Refs. 7 and 8). However, neither the rglo nor the hglo intron was efficiently spliced when placed in the foreign environment of firefly luciferase exon 1. The failure of either of the β-globin introns to be spliced when placed in the luciferase exon is consistent with other studies indicating a role for flanking exon sequences and splice site context in splicing of constitutively expressed transcripts (29, 30) as well as alternatively spliced transcripts (31, 32). The lack of context dependency of the adenovirus intron could be due to the close match of its 5′ SS, BP, and 3′ SS to the ideal consensus sequences at these sites.

Alteration of these elements to the consensus sequences was all that was required to increase splicing of β-globin transcripts to the same high level as observed for the adenovirus pre-mRNAs. The 5′SS, BP, and 3′ SS act cooperatively to promote splicing because simultaneous improvement of all three sites was required for maximal splicing efficiency. Likewise, mutation of all three elements in the adenovirus intron was required for elimination of splicing of these transcripts. Results from our study suggest that strong consensus splicing signals remove the context dependency of splicing and that, in the absence of strong splicing elements, meaningful rates of splicing can be observed only in the context of the natural environment of the intron. Moreover, an informed guess regarding the rate-limiting introns in a multi-intron pre-mRNA can be made based on analysis of the intron sequences at the 5′SS, BP, and 3′SS.

To confirm the usefulness of the assay and the validity of the predictions based on analysis of factors that influence the extent of splicing, we studied the ability of antisense oligonucleotides to inhibit splicing of two luciferase reporters that spliced with different efficiencies. Before screening antisense oligonucleotides, we performed experiments to ensure that the effects observed were due to sequence-specific antisense effects and not non-sequence-dependent effects on cell viability (data not shown). Although calcium phosphate-mediated transfection efficiency is enhanced by glycerol treatment of the cells, we found that elimination of this step enhanced viability when cells were also treated with oligonucleotides. For maximal cell viability, it was important to minimize the time during which cells were incubated with antisense oligonucleotides and transfected DNA and the time between oligonucleotide treatment and the luciferase assays. To differentiate between sequence-specific and nonspecific effects, it was critical to use relatively low oligonucleotide and lipofectin concentrations. Oligonucleotide specificity was also deter-

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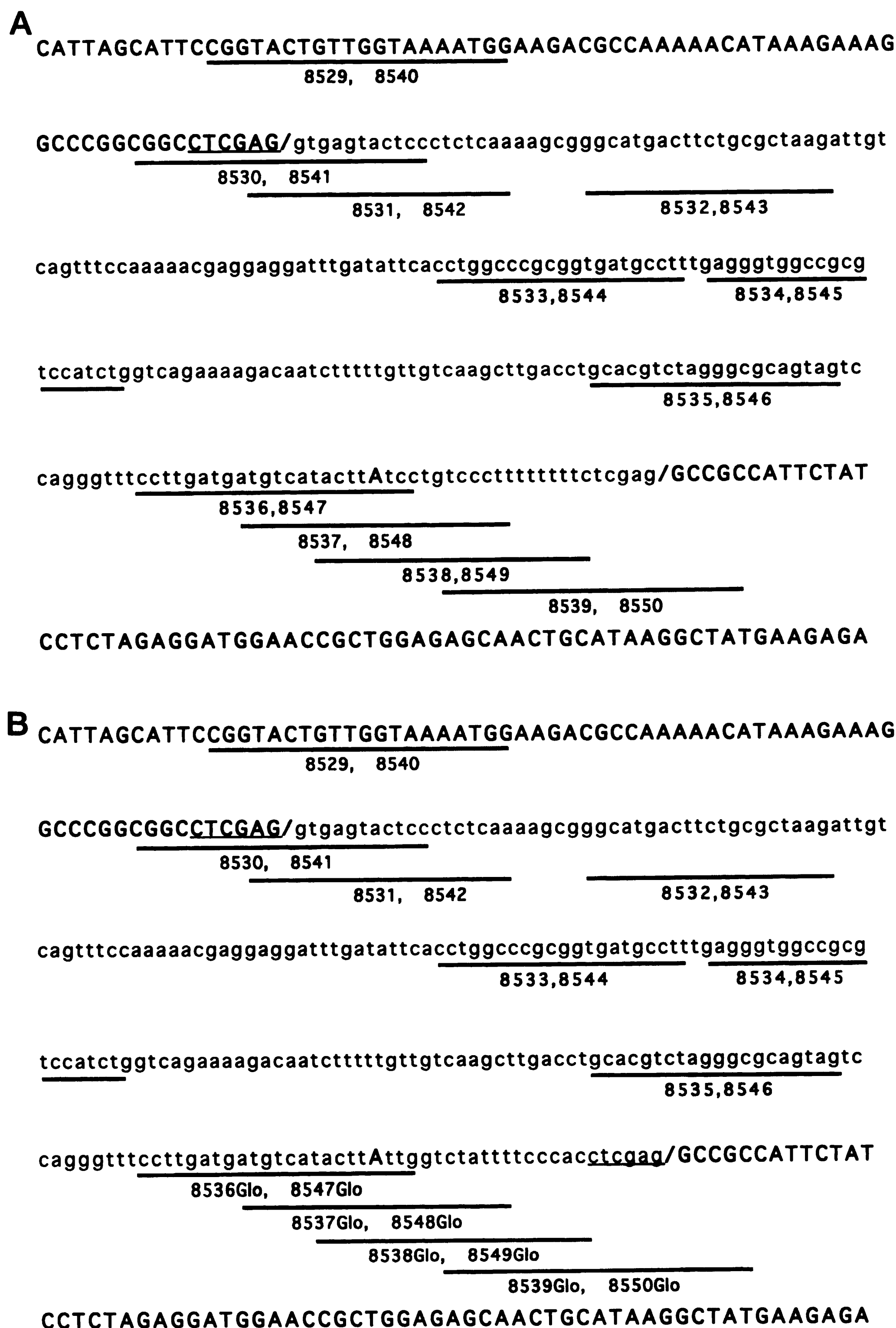

Fig. 5. A, Sequence of the wild-type LI-12 adenovirus intron region targeted by antisense oligonucleotides. *Bold uppercase*, luciferase coding region. Lowercase, wild-type adenovirus intron. Regions targeted by antisense oligonucleotides are *underlined*, with the identity of the deoxy and 2′ methoxy oligonucleotides, respectively, shown below. The antisense oligonucleotide sequences are shown in Table 2. B, Sequence of the LI-12 adenovirus hglo 3/intron region targeted by antisense oligonucleotides. The adenovirus intron sequence is the same as shown in Fig. 5A except for the 3′ SS region, which has been mutated to the sequence found in the hglo intron. Sequence and oligonucleotide designations are as shown in Fig. 5A.

mined by analyzing the effect of control oligonucleotides complementary to sequences not present in the luciferase pre-mRNA. Dose-response curves were critical for determining the potency and specificity of the antisense effects and direct analysis of the RNA from treated cells was essential for confirming that differences in luciferase activity were actually due to effects on splicing.

To determine if antisense oligonucleotide binding alone (competition with or displacement of splicing factors) could inhibit splicing or if RNase H-mediated cleavage of pre-mRNA/oligonucleotide duplexes was required for inhibitory activity, we compared the effects of deoxy and 2′ methoxy phosphorothioate oligonucleotides. We used 2′ methoxy oligonucleotides to avoid the complexity of RNase H-mediated degradation of RNA and, therefore, to evaluate the effects of binding of an oligonucleotide to the target RNA only. Neither

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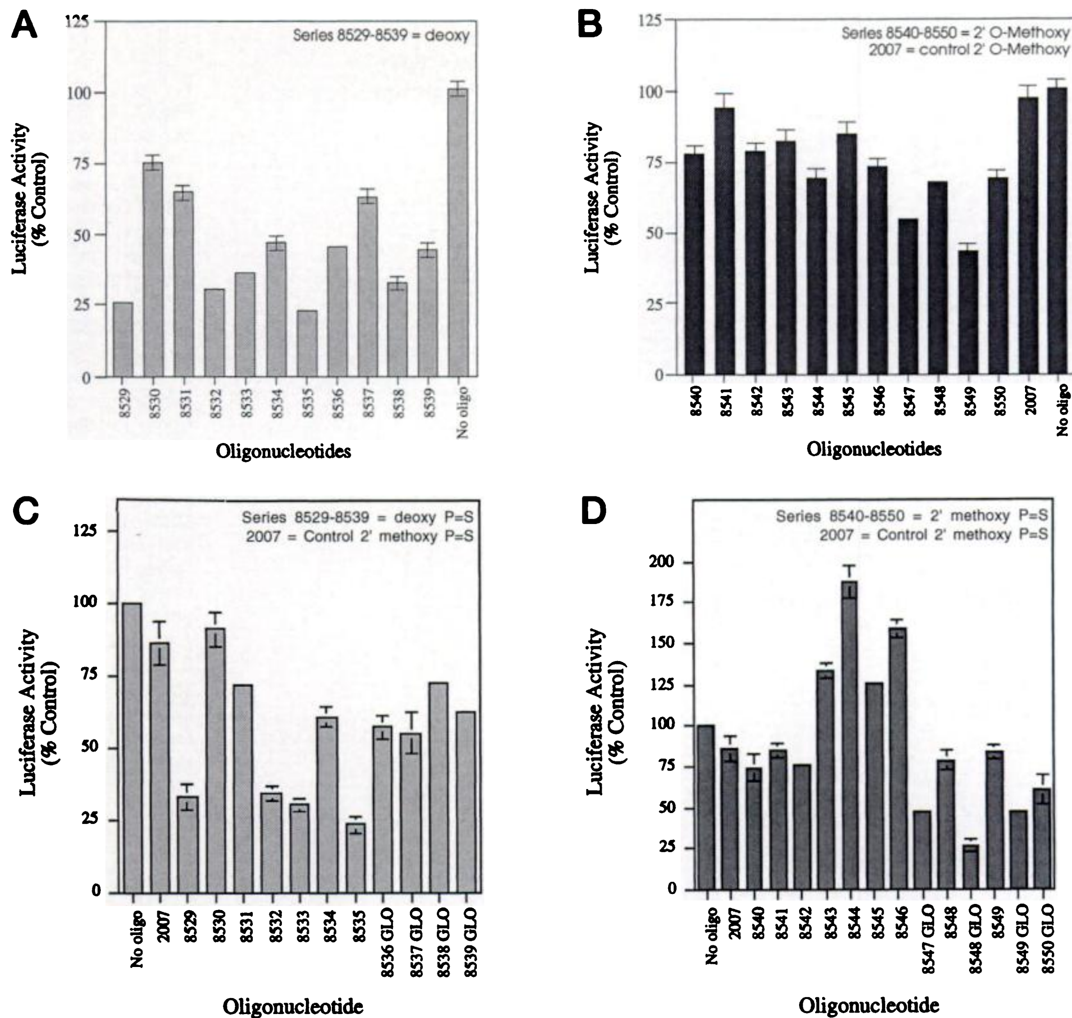

Fig. 6. A, Inhibition of wild-type LI-12 adenovirus expression by phosphorothioate oligodeoxynucleotides. HeLa cells were treated with antisense oligonucleotides at a concentration of 150 nM for 2 hr before transfection with LI-12 Ad(+). Luciferase activity is shown as a percentage of the control (transfection and treatment with lipofectin but without antisense oligonucleotides) value. Standard error is shown for values obtained from triplicate transfections. B, Inhibition of wild-type LI-12 adenovirus expression by phosphorothioate 2/methoxy oligonucleotides. Transfections and antisense oligonucleotide treatments were performed as described in A except the antisense oligonucleotides were 2′ methoxy derivatives. C, Inhibition of LI-12 adenovirus hglo 3/expression by phosphorothioate oligodeoxynucleotides. HeLa cells were treated with phosphorothioate oligodeoxynucleotides at a concentration of 150 nM for 2 hr before transfection with LI-12 adeno-hglo 3′. Luciferase analyses are described in the legend to A. D, Inhibition of LI-12 Ad-hglo 3/expression by phosphorothioate 2/methoxy oligonucleotides. Transfections, antisense oligonucleotide treatments, and luciferase analyses were performed as described in the legend to C except that the antisense oligonucleotides were 2′ methyl derivatives.

class of oligonucleotides targeted to the 5′ SS of either splicing reporter substantially inhibited splicing. This is in contrast to *in vitro* studies that showed that high concentrations (4 μM) of 2′ methoxy oligonucleotides, which targeted the 5′ SS of the hglo intron inhibited splicing (25). Our results suggest that the 5′ SS of the adenovirus intron is inaccessible to antisense attack or that the currently available antisense oligonucleotides are unable to compete effectively with snRNPs for binding to this region *in vivo*. In addition to differences in the oligonucleotide concentrations and targets, differences between *in vitro* and *in vivo* results are likely due to differences in the abundance of snRNPs and other splicing proteins, as well as structural differences in the pre-mRNA in the two systems. Base-pairing of U1, U6, and U5 snRNA with the 5′ SS occurs early in spliceosome formation, and the ability of antisense oligonucleotides to inhibit these interactions will depend on the strength (match to the consensus sequences) of the 5′ SS. Because these interactions are enhanced by the cooperative interactions of snRNPs bound at the 3′ SS and 5′ SS (33), the strength of the 3′ SS will also affect the ability of antisense oligonucleotides targeted to the 5′ SS to inhibit splicing.

Phosphorothioate oligodeoxynucleotides that can support RNase H cleavage were better inhibitors of expression of the wild-type adenovirus construct than were the 2′ methoxy phosphorothioates that cannot support RNase H. This was especially true for antisense oligonucleotides that targeted the region between the 5′ SS and BP. The 2′ methoxy oligonucleotides targeted to this region (ISIS 8543–8546) would not be expected to inhibit the binding of splicing factors because this region has not been shown to participate in spliceosome assembly. The potent inhibition by the oligodeoxynucleotides indicates that this region is available for formation of RNA/DNA duplexes, which can serve as substrates

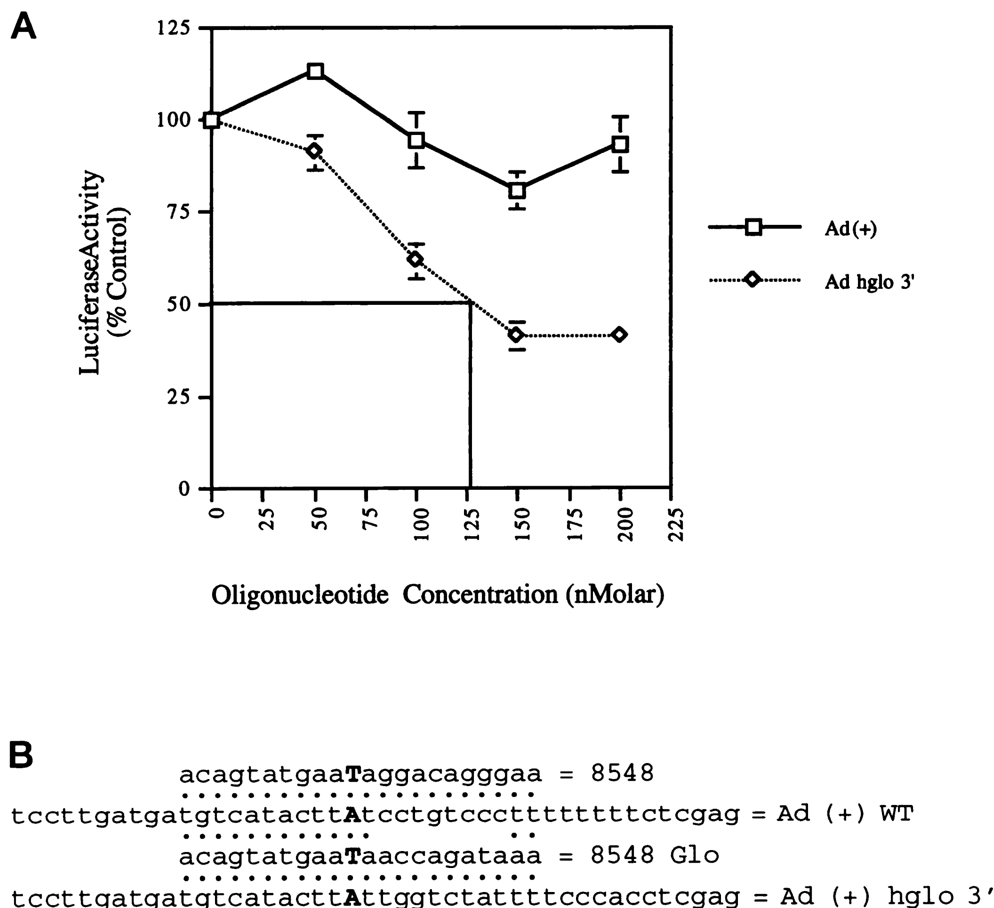

Fig. 7. A, 8548 Glo dose-response curve showing comparison of inhibition of LI-12 Ad-hglo 3/and wild-type LI-12 Ad. HeLa cells were pretreated with increasing concentrations of 2′ methoxy oligonucleotide 8548 Glo followed by transient transfection with wild-type LI-12 Ad (□) or LI-12 Ad-hglo 3′ (◇). The oligonucleotide/lipofectin ratio was maintained at 100 nM oligonucleotide/1.0 μg/ml lipofectin. Luciferase activity is shown as a percentage of the values obtained from lipofectin but no oligonucleotide treatment. The oligonucleotide concentration that decreased luciferase activity to 50% of the control value is shown by *horizontal and vertical lines that intersect with the Ad-hglo 3′ curve*. B, Comparison of the complementarity of antisense oligonucleotides 8548 and 8548 Glo to LI-12 Ad(+) wild-type and LI-12 Ad(+) hglo 3′. *Dots*, sequences of the two antisense oligonucleotides and the two luciferase reporters are aligned with nucleotide complementarity. Note that 8548 Glo is completely complementary to Ad(+) hglo 3′ but mismatched at 8 of 22 nts with Ad(+) wild-type.

for RNase H cleavage. However, as we did not examine the effects of these oligonucleotides on the pre-mRNA levels, we have not proved that cleavage of the pre-mRNA induced by RNase H occurred. Although the oligodeoxynucleotides targeted to this region of the mutated Ad wild-type 5′-hglo 3′ pre-mRNAs inhibited luciferase expression quite well, the 2′ methoxy oligonucleotides enhanced expression (Fig. 6D). RT-PCR analysis confirmed that splicing was actually affected because the luciferase pre-mRNA decreased and the mRNA increased with ISIS 8544 and ISIS 8548 treatment. Because the pre-mRNA in the region targeted by oligonucleotides 8543–8546 is the same for both luciferase reporters, the different effects of these oligonucleotides are likely due to differences in RNA structure or pre-mRNA/snRNP interactions induced by alterations at the 3′ SS. A role for RNA structure in modulating splicing has been demonstrated previously (for a review, see Ref. 8).

Although oligodeoxynucleotides targeted to the BP and 3′ SS of the wild-type adenovirus intron were better inhibitors of luciferase expression than were the 2′ methoxy oligonucleotides, the reverse was true for oligonucleotides targeted to the BP and 3′ SS of adenovirus wild-type 5′-hglo 3′ transcripts. The 2′ methoxy oligonucleotide, ISIS 8548 GLO, which is centered over the modified BP, was especially effective at inhibiting splicing of these transcripts. This suggests that the weaker BP-3′ SS in the AD-hglo 3′ pre-mRNAs increases the ability of the 2′ methoxy oligonucleotides to compete with the snRNPs or other proteins for binding to this region. Because the BP sequence and location are highly degenerate in mammals and the U2 snRNA/pre-mRNA interaction involves only a few nucleotides, other proteins are likely to be required to stabilize U2-snRNA/BP interactions. Recently, a spliceosome-associated protein, SAP 49, in combination with SAP 145 was found to associate specifically with U2 snRNP and to bind directly to the pre-mRNA immediately upstream of the BP sequence in the adenovirus intron (34). This protein complex was proposed to assist in the binding of U2-snRNP to the BP. ISIS 8548 and 8548 GLO are targeted to the same region and, presumably, must compete with SAP49/145 and U2-snRNP for binding. Our results suggest that the antisense oligonucleotides analogues that we tested are unable to compete efficiently with these proteins for binding to their optimal recognition sequences in the pre-mRNA of efficiently spliced introns. Clearly, higher affinity analogues should be tested, e.g., 2′ fluoro. However, naturally occurring modifications at this site, such as those found in the β-globin introns, should bind these proteins less well and, thus, increase the accessibility of antisense oligonucleotides to these pre-RNAs.

Our studies suggest that phosphorothioate oligodeoxynucleotides that can support RNase H cleavage of the target RNAs are the best inhibitors of efficiently processed pre-mRNAs such as our artificial adenovirus substrate. However, less efficiently spliced transcripts are potential targets for modified phosphorothioate oligonucleotides that inhibit splicing by competing with splicing factors for binding to the pre-mRNA. For the adenovirus intron with a mutated 3′ SS, we found that there was a hierarchy of 2′ methoxy antisense oligonucleotide effects with the BP being the best target for inhibition. The two artificial splicing substrates we tested differed in splicing efficien-

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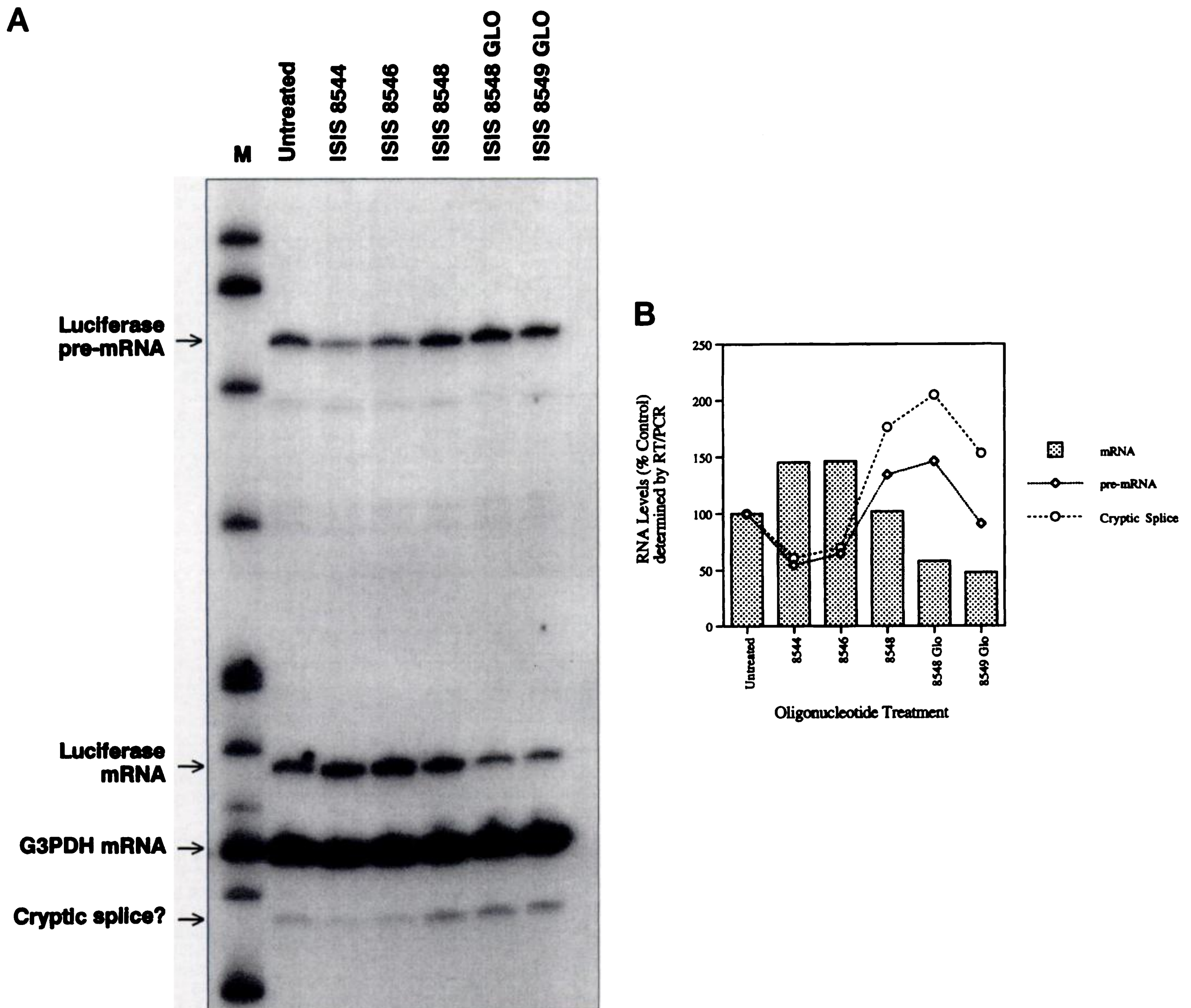

Fig. 8. A, Assay of antisense oligonucleotide inhibition of expression of LI-12 Ad(+) hglo 3 by RT-PCR. RNA was purified from HeLa cells after antisense oligonucleotide treatments (150 nM) and transfection with LI-12 Ad(+) hglo 3′. RT-PCR and gel analyses were performed as described in the legend for Fig. 3B except that PCR amplification was limited to 25 cycles and autoradiography was performed for 41 hr at −80° with an intensifying screen. B, Phosphorimager quantification of RT-PCR products. The RNA species on the polyacrylamide gel shown in A were quantified using a phosphorimager. The luciferase pre-mRNA, mRNA, and presumed cryptic splice values were normalized to G3PDH levels and plotted as the percentage of the control values (untreated = lipofectin but no oligonucleotide).

cies but, nevertheless, spliced quite well. It was, thus, not surprising that oligonucleotides targeted to intron sequences were no more effective than those targeted to exon sequences in inhibiting luciferase expression. However, we suggest that *in vivo* targets may exist whose expression will be inhibited more effectively by antisense oligonucleotides that target the pre-mRNA rather than the mRNA. A hallmark of alternatively spliced introns is weak splicing elements at the 5′ SS, BP, and/or 3′ SS. This makes these introns attractive targets for antisense oligonucleotides. Based on our results, we make the following suggestions for selection of antisense targets: (a) select introns for antisense attack that lack strong consensus splicing sequences and may, therefore, represent the rate-limiting intron in splicing of the pre-mRNA, (b) select introns with nonconsensus 3′ SS BP and polypyrimidine tracts, and (c) select oligonucleotides that serve as substrates for RNase H, if possible. We also suggest that alternatively spliced transcripts with nonconsensus splice sites are attractive targets for antisense drug development.

Although we have not tested this directly, we hypothesize that splicing may be redirected through the binding of non-RNase H activating oligonucleotides that displace splicing factors or alter RNA secondary structures required for an undesirable splicing pathway and, thereby, promote use of alternative pathways. Finally, the observation that in some cases oligonucleotides that do not serve as substrates for RNase H may enhance production of a target protein may be quite important as it suggests the possibility that antisense oligonucleotides may be designed to increase rather than inhibit production of a therapeutically important target.

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Acknowledgments

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