

# Deletion of Individual Exons and Induction of Soluble Murine Interleukin-5 Receptor- $\alpha$ Chain Expression through Antisense Oligonucleotide-Mediated Redirection of Pre-mRNA Splicing

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## ABSTRACT

Expression of the interleukin-5 receptor- $\alpha$  (IL-5R $\alpha$ ) chain is thought to play an important role in the pathogenesis of asthma and other eosinophilic diseases. With antisense oligonucleotides (ASOs) chemically modified to provide increased hybridization affinity for RNA but that do not support RNase H-mediated cleavage (2'-O-methoxyethyl-modified ASOs), we show that constitutive splicing of murine IL-5R $\alpha$  mRNA can be modulated in cells such that individual exons may be selectively deleted from mature transcripts. Specific deletion of individual exons and redirection of alternative splicing of the IL-5R $\alpha$  mRNA have been achieved with this approach, by targeting 3'-splice sites or exon sequences immediately downstream of an alternative splice site. ASO targeting with these strategies

resulted in inhibition of mRNA and protein levels of the membrane IL-5R $\alpha$  isoform capable of signaling IL-5-mediated growth and antiapoptotic signals to eosinophils. Membrane isoform IL-5R $\alpha$  inhibition was coupled with an increase in expression of mRNA for the alternatively spliced soluble isoform, which binds IL-5 extracellularly and may block its function. These observations suggest the potential general therapeutic use of an antisense approach to increase expression of variant RNA transcripts and to thereby produce proteins devoid of specific functional domains that may impact disease processes, as well as its specific utility for modulating expression of a key cytokine receptor implicated in allergic inflammation.

Antisense oligonucleotides (ASOs) have received increased attention due to their potential for treating human disease (Crooke, 1992; Stein and Cheng, 1993; Crooke and Bennett, 1996). DNA-phosphorothioate and "second generation" chemically modified oligonucleotides have been shown to provide pharmacological benefit and reduce target gene expression in vivo (Bennett et al., 1995; Altmann et al., 1996; Monia, 1997; Orr and Monia, 1998). The chemical modifications incorporated into antisense design have been driven by the need to increase nuclease resistance as well as improve hybridization efficiency and pharmacokinetic profile. Although ASOs entered in clinical trials to date have been designed to support enzyme-mediated mechanisms of RNA degradation, continued interest has been directed to the development of novel mechanisms of oligonucleotide interference with gene expression.

The regulation of signal transduction through cytokine receptors in vivo is complex and is determined in part by relative expression of different isoforms that arise from alternative splicing. In the murine interleukin-5 receptor- $\alpha$  (IL-5R $\alpha$ ) gene, there is evidence that soluble forms of the receptor arise by exclusion of exon 9, the transmembrane

coding region, or alternatively, of exon 9 and exon 10 (Imamura et al., 1994). These soluble receptor proteins can bind and neutralize IL-5 bioactivity, presumably by preventing interaction of the cytokine with the membrane-bound form of the receptor  $\alpha$ -chain (Kikuchi et al., 1994; Takatsu et al., 1994). Only the membrane form is complexed with the common  $\beta$ -chain, which is required for signaling and also forms heterodimers with IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF)  $\alpha$ -chains (Hara and Miyajima, 1992; Park et al., 1992). Thus, expression of the soluble forms of IL-5R $\alpha$  may be beneficial in disease states where IL-5 has been shown to play a prominent role, such as eosinophilic syndromes and asthma.

It has been previously shown that chemically modified ASOs can induce correction of aberrantly spliced mutant human  $\beta$ -globin constructs stably expressed in mammalian cells (Sierakowska et al., 1996). To determine whether selective modulation of endogenous constitutive or alternative mRNA splicing in cells could be achieved with an ASO approach, we designed uniformly 2'-O-methoxyethyl (MOE)-modified ASOs to sequences either within the alternatively spliced exon 9 or across various intron-exon boundaries in

**ABBREVIATIONS:** ASO, antisense oligonucleotide; IL-5R $\alpha$ , interleukin-5 receptor- $\alpha$ ; GM-CSF, granulocyte-macrophage colony stimulating factor; 2'-MOE, 2'-O-methoxyethyl; RPA, RNase protection assay.

the mouse IL-5R $\alpha$  gene and tested their activity in the IL-5R $\alpha^+$  mouse BCL $_1$  B cell lymphoma. The patterns of IL-5R $\alpha$  mRNA expression were then examined with Northern blotting and an RNase protection assay with probes that distinguish the membrane and soluble forms of the receptor mRNA as well as individual exons within the gene. With this strategy, oligonucleotides were discovered that both selectively inhibited expression of the membrane form of the IL-5R $\alpha$  and simultaneously superinduced transcript levels of the soluble form. Additionally, specific loss of the immediate downstream exon was observed with oligonucleotides targeted to several different 3'-splice sites within the mouse IL-5R $\alpha$  pre-mRNA, suggesting that a rational strategy of targeted exon deletion in mRNA transcripts may be possible with chemically modified ASOs.

## Materials and Methods

**Cell Culture.** The B lymphoma cell line BCL $_1$  was purchased from the American Type Culture Collection (Rockville, MD). BCL $_1$  cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO); 10 mM HEPES, pH 7.2; 50  $\mu$ M 2-mercaptoethanol; 2 mM L-glutamine; 100 U/ml penicillin; and 100  $\mu$ g/ml streptomycin (Life Technologies, Grand Island, NY).

**ASO Synthesis and Cell Transfection.** 2'-O-Methoxyethylribose-modified phosphorothioate oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) as described previously (Monia et al., 1992). Both uniformly 2'-MOE-modified and chimeric oligonucleotides were used in these studies (see Table 1). The chimeric oligonucleotides contain 2'-MOE-modified residues flanking a 2'-deoxynucleotide/phosphorothioate region (gap) that supports RNase-H activation (Monia et al., 1993). Oligonucleotides were analyzed by capillary gel electrophoresis and judged to be at least 85% full-length material. BCL $_1$  cells ( $1 \times 10^7$  cells in PBS) were transfected with oligonucleotides by electroporation at 200 V, 1000  $\mu$ F with a BTX Electro Cell Manipulator 600 (Genetronics, San Diego, CA).

**Northern Blotting and Probe Design.** Total cellular RNA was isolated with the RNeasy kit (Qiagen, Santa Clarita, CA). Northern blotting was performed as previously described (Monia et al., 1992) with cDNA probes generated from oligonucleotides matching the exon sequences of either exons 8, 9, or 10. Signals were quantitated with a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

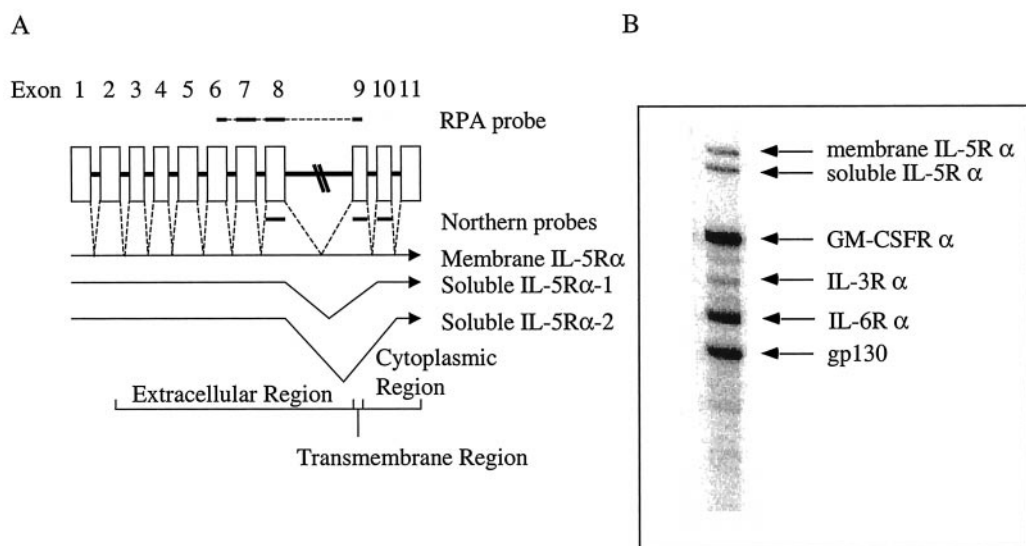
**Riboprobe Design and RNase Protection Assay.** RNase protection experiments were conducted with Riboquant kits according to the manufacturer's instructions (Pharmingen, San Diego, CA). A custom riboprobe was designed to protect the mRNA sequence corresponding to the distal half of exon 6, all of exons 7 and 8, and the proximal half of exon 9, and purchased from Pharmingen. Signals were quantitated with a Molecular Dynamics PhosphorImager.

**Western Blot Analysis.** Western blotting was performed as described previously (Dean and McKay, 1994). For Western blotting, membrane-enriched fractions were prepared as Triton X-100 insoluble material (Dean and McKay, 1994) and separated by SDS-polyacrylamide gel electrophoresis with 8% gels. Antibody to mouse IL-5R $\alpha$  was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:1000 dilution for Western blotting.

## Results

**RNase H-Dependent ASOs Do Not Differentially Inhibit Murine IL-5R $\alpha$  mRNA Membrane and Soluble Isoforms.** Exon 9 of the murine IL-5R $\alpha$  chain represents the only nucleotide sequence unique to the membrane form transcript and encodes the transmembrane domain (Imamura et al., 1994). Evidence indicates that two soluble forms of IL-5R $\alpha$  mRNA are produced by alternative splicing events, splicing exon 8 to exon 10 or 11, respectively (Takaki et al., 1990; Imamura et al., 1994; Fig. 1A).

The murine B cell lymphoma BCL $_1$  expresses mRNA for the membrane and soluble forms of the IL-5R $\alpha$  chain, as analyzed by RNase protection assay (RPA; Fig. 1B; see Fig. 1A for probe placement). Because the RPA probe does not extend to exon 10 sequence, only one species of soluble receptor mRNA was observed in RPA analysis of BCL $_1$  RNA. To



**Fig. 1.** Schematic of alternative splicing of the murine IL-5R $\alpha$  chain gene and analysis of membrane and soluble receptor mRNA species by RNase protection assay. A, arrows show how splicing yields both membrane and soluble forms of mouse IL-5R $\alpha$ . Although not shown, removal of introns occurs in soluble receptor isoforms similarly to that shown for membrane receptor. The locations of the RNase protection and Northern probes are shown above and below the gene structure, respectively. B, representative RPA profile of BCL $_1$  RNA, showing approximately equal expression of membrane and soluble receptor mRNA in untreated cells. Because the probe does not extend into exon 10, both species of soluble receptor mRNA have the same length. Additional probes generated from the template set also detect expression of mRNA for GM-CSF receptor- $\alpha$ , IL-3 receptor- $\alpha$ , IL-6 receptor- $\alpha$ , and gp-130 in BCL $_1$  cells.

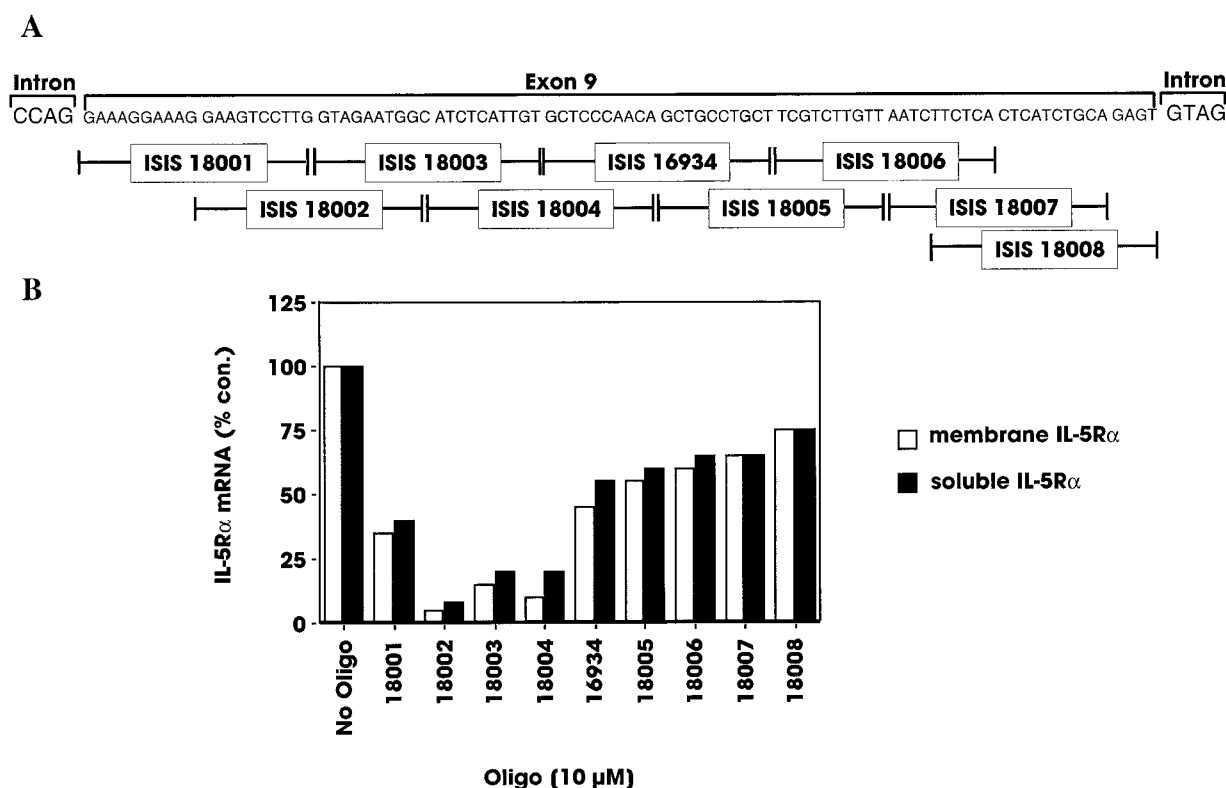
develop an ASO that specifically inhibits the membrane-bound form of the receptor, a series of 20mer 2'-MOE ribonucleotide "gapmers" were synthesized, overlapping every 10 nucleotides across the 90-base pair exon (Fig. 2A). These ASOs contain uniform phosphorothioate backbones and a stretch of 10 2'-deoxy residues in the center of the molecule that supports RNase H cleavage flanked by five bases at each of the 5' and 3' ends that are MOE modified and thus provide greater stability against exonuclease activities and higher affinity for hybridization to RNA (Monia et al., 1993; Altmann et al., 1996; Dean and Griffey, 1997). Oligonucleotides containing uniform 2'-MOE modification have previously been shown to be poor substrates for RNase H-mediated cleavage in cells (Monia et al., 1993). The exon 9 spanning ASOs were electroporated into BCL<sub>1</sub> cells and RPA was used to examine inhibition of membrane and soluble IL-5R $\alpha$  mRNA. As shown in Fig. 2B, several of the oligonucleotides reduced mRNA levels but none showed specificity for the membrane isoform, suggesting that the pre-mRNA species is targeted.

**Targeting 3'-Splice Sites with Uniformly 2'-MOE-Modified ASOs Induces Specific Exon Deletion.** In a second attempt to selectively inhibit the membrane-bound isoform of IL-5R $\alpha$ , we compared the effects of a 3'-splice site targeting approach, with uniformly 2'-MOE-modified ASOs, on splicing of exons 9 and 10, as well as the constitutively spliced exon 8. Because only four bases of intron sequence are known flanking exons 8, 9, and 10 of the mouse IL-5R $\alpha$  gene (Imamura et al., 1994), uniform 2'-MOE ASOs were synthesized that would hybridize to these four bases and the ad-

joining 16 bases of exon sequence at the 3'-splice site. In contrast to MOE gapmers, these oligonucleotides do not support RNase H activity but exhibit enhanced hybridization affinity for target mRNA (Monia et al., 1993; Altmann et al., 1996).

To evaluate whether bridging a 3'-splice site with these uniformly 2'-MOE-modified ASOs induces specific alterations in splicing activity, we generated individual cDNA probes specific for either exons 8, 9, or 10 and performed Northern blots on RNA from transfected BCL<sub>1</sub> cells. An exon 9 2'-MOE gapmer shown to be active in Fig. 2 (ISIS 18002) was used as a control to show that an ASO that supports RNase H activity induces degradation of the IL-5R $\alpha$  transcript that is reflected by all three probes (Fig. 3). Importantly, transfection with uniformly modified 2'-MOE oligonucleotides targeted to the 3'-splice sites of the introns preceeding exons 8, 9, or 10 resulted in specific exclusion of that respective downstream exon but not that of the other two following, adjacent, or upstream exons. Hence, targeting the 3'-splice site intron/exon junctions with high-affinity ASOs can yield selective deletion of individual exons within the mRNA transcript, including exclusion of an exon that is constitutively spliced.

To determine whether targeting of the 5'-splice site would result in similar effects on splicing of IL-5R $\alpha$ , a uniform 2'-MOE ASO was designed for the 5'-intron 8 splice site. After transfection of BCL<sub>1</sub> cells with oligonucleotides hybridizing to either the 3'- or 5'-splice sites, mRNA was prepared and analyzed by RPA. Treatment with the 3'-splice site-targeted oligonucleotide resulted in loss of the full-length

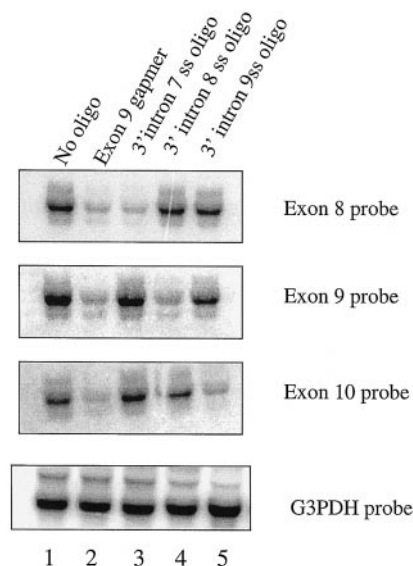


**Fig. 2.** Lack of selectivity of 2'-MOE gapmers targeted to exon 9 for the membrane-bound isoform of the murine IL-5R $\alpha$ . A, positions of ASOs on the exon 9 sequence. The bracketed sequences represent exon 9 and available flanking intron sequences. ISIS 16934-18008 are 2'-MOE "gapmer" modified, RNase H-dependent ASOs. B, quantitation of RPA phosphorimage analysis of effects of 2'-MOE gapmer ASOs depicted in A on membrane (open bars) and soluble (shaded bars) IL-5R $\alpha$  mRNA levels in BCL<sub>1</sub> cells. The data shown are derived from analysis of RPA gel 24 h after electroporation with ASOs. The data are normalized to L32 levels.



membrane and soluble IL-5R $\alpha$  bands and the appearance of a new band of smaller size (Fig. 4, lanes 4 and 5). Because the RPA probe spans part of exon 6, all of exons 7 and 8, and part of exon 9, this novel band most likely corresponds to the protected fragment constituting sequence from exons 6 and 7. This result suggests that treatment with this oligonucleotide produces a transcript that lacks exon 8 and that the protected fragment of exon 9 is too small to be visualized on the gel. These results are in agreement with Northern data shown in Fig. 3. In contrast, treatment with the 5'-intron 8 splice site oligonucleotide partially inhibited full-length membrane and soluble IL-5R $\alpha$  transcripts and produced another novel mRNA species of a size in agreement with the induced selection of a cryptic splice site within exon 8 (Fig. 4, lanes 6 and 7). Thus, our data suggest that placement of an ASO with high affinity for its target mRNA sequence on a 5'-splice site can generate a novel mRNA transcript that results from use of a nearby upstream cryptic splice site.

**ASOs targeted to the 3'-Splice Site of Exon 9 Modulate Alternative Splicing of Murine IL-5R $\alpha$ .** To more fully address optimal placement of uniform 2'-MOE ASOs to modulate alternative splicing of mouse IL-5R $\alpha$ , additional phosphorothioate 20mers were designed across the 5'-splice site of exon 9, as well as to the same target sequences tested previously as MOE chimeric gapmers (Fig. 2A). The activities of these ASOs in BCL<sub>1</sub> cells were then assessed by RPA to directly compare effects on expression of the membrane and soluble receptor isoforms. Strikingly, electroporation of BCL<sub>1</sub> cells with these oligonucleotides not only resulted in reduced levels of the membrane form of IL-5R $\alpha$  mRNA but also in the concomitant superinduction of expression of soluble form transcript (Fig. 5). Although these activities were observed for all of the oligonucleotides targeting exon 9 and the known intron sequence, the potencies of both of these concurrent

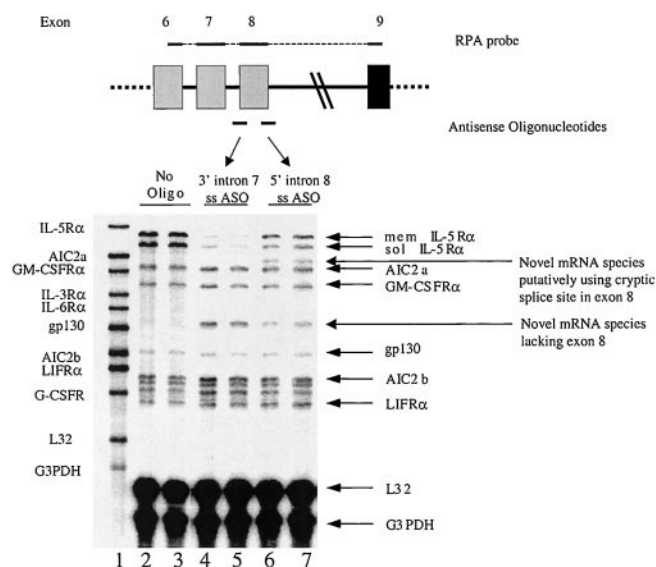


**Fig. 3.** Specific exclusion of individual exons of the IL-5R $\alpha$  pre-mRNA with uniformly 2'-MOE-modified ASOs targeting the respective 3'-splice sites. Northern blots of RNA prepared from BCL<sub>1</sub> cells electroporated in the presence of no oligonucleotide (lane 1); ISIS 18002 (the active exon 9 2'-MOE gapmer, lane 2); or uniformly 2'-MOE-modified oligonucleotides (10  $\mu$ M) that hybridize across the 3'-intron 7 splice site preceding exon 8 (lane 3), the 3'-intron 8 splice site preceding exon 9 (lane 4), or the 3'-intron 9 splice site preceding exon 10 (lane 5). The cDNA probes used were specific for exon 8, 9, or 10, as indicated on the right.

effects were coordinately diminished by moving the targeted sequence toward the 5'-splice site. The oligonucleotide used in the Northern blotting experiments shown in Fig. 3 (ISIS 21750) also inhibited membrane IL-5R $\alpha$  expression by RPA, as expected; however, ASOs targeting nearby exon sequences were of similar potency. These data suggest that targeting of the 3'-splice site, or of sequences immediately downstream and completely within the alternatively spliced exon 9 of the murine IL-5R $\alpha$  mRNA, with a uniformly 2'-MOE modified ASO results in redirection of splicing activity, such that membrane IL-5R $\alpha$  mRNA expression is inhibited whereas levels of the soluble form are increased.

The sequence specificity of one of the most potent of these exon 9 uniformly modified oligonucleotides, ISIS 21752, was examined in a dose-response analysis with control oligonucleotides in which 1, 3, or 5 base mismatches were incorporated. As shown in Fig. 6A, membrane form mRNA levels were dose dependently decreased by ISIS 21752, whereas the successive incorporation of mismatches resulted in a graded loss of activity of the overall sequence. The increase in soluble receptor mRNA from basal levels induced by treatment with ISIS 21752 also was dose-dependent and was correspondingly reduced by incorporating base mismatches (Fig. 6B). Thus, the effects of ISIS 21752 on IL-5R $\alpha$  mRNA expression are consistent with a hybridization-dependent mechanism.

In addition, studies were performed to evaluate the kinetics of redirection of splicing and the duration of oligonucleotide action. Inhibition of membrane form mRNA as well as superinduction of soluble form transcript were observed as early as 4 h after transfection of ISIS 21752 (Fig. 6C). These



**Fig. 4.** Modulation of splicing and splice site use in IL-5R $\alpha$  chain pre-mRNA by uniformly modified 2'-MOE ASOs. A, schematic of ASO targeting strategy, showing positions of uniformly 2'-MOE-modified oligonucleotides and region covered by RPA probe. B, RPA analysis of membrane and soluble IL-5R $\alpha$  mRNA species after transfection of BCL<sub>1</sub> cells with uniformly modified 2'-MOE ASOs (10  $\mu$ M) targeting either the 3'-intron 7 (lanes 4 and 5) or the 5'-intron 8 (lanes 6 and 7) splice sites. Arrows indicate the novel mRNA species generated on oligonucleotide treatment. RNA from cells that were electroporated in the absence of oligonucleotide is shown in lanes 2 and 3. Undigested probe is shown in lane 1. The data shown are representative of three individual experiments. AIC2a and AIC2b, homologous  $\beta$ -subunits of IL-3, IL-5, and GM-CSF receptors; LIFR $\alpha$ , leukemia inhibitory factor receptor  $\alpha$ .

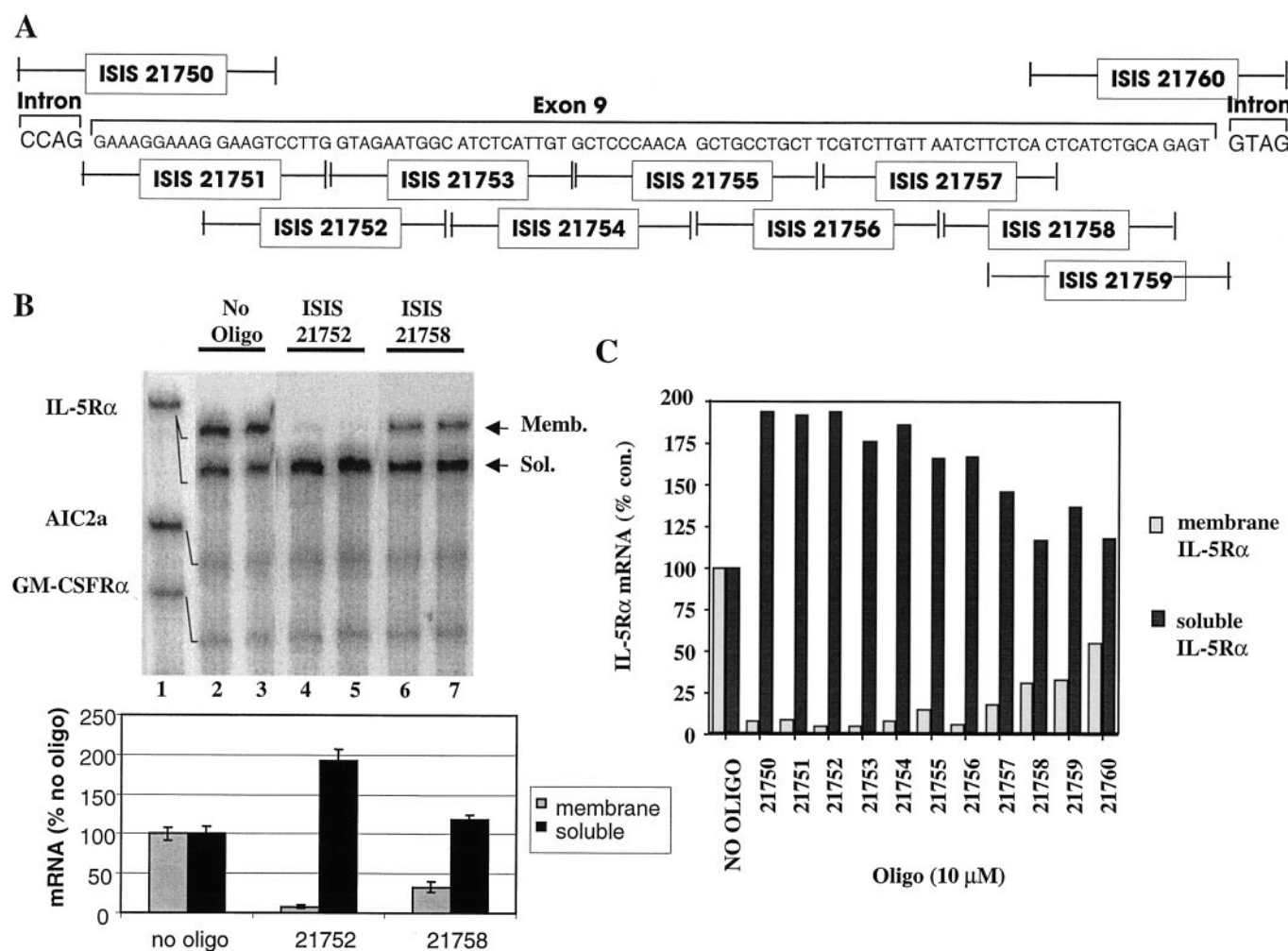
activities of the oligonucleotide were prolonged, with a similar magnitude of effect lasting out to 72 h post-treatment.

Inhibition of membrane form mRNA correlated with subsequent reduction of membrane form IL-5R $\alpha$  protein levels because ISIS 21752 was found to diminish the ~60-kD form of IL-5R $\alpha$  in Triton X-100 insoluble membrane fraction-enriched cell extracts from BCL<sub>1</sub> cells as determined by immunoblotting (Fig. 7). Control ASOs, targeted at sequences present only in processed soluble isoform mRNA (bridging exon 8 to exon 10, ISIS 21853, or exon 8 to exon 11, ISIS 21855) did not produce inhibition of membrane IL-5R $\alpha$  protein (lane 4), suggesting specificity of action of ISIS 21752. As supporting evidence that the observed ~60-kD band represents the IL-5R $\alpha$  membrane-bound form, an antisense inhibitor that targets sequence within the 3'-untranslated region and hence inhibits all forms of the IL-5R $\alpha$  mRNA (ISIS 16949) also reduced expression of this band. Soluble IL-5R $\alpha$  was not detectable in this assay (data not shown) and has not historically been measurable in vitro or in vivo without the

use of recombinant DNA techniques (Kikuchi et al., 1994; Takaki et al., 1990). However, an antisense approach similar to that described in our work targeting exon sequence near the 5'-splice site of the *bcl-x* pre-mRNA produced increased expression of bcl-x<sub>s</sub> protein (Taylor et al., 1999), indicating that this strategy can result in up-regulation of mRNA and its corresponding protein product.

## Discussion

We have observed that targeting 3'-splice sites of the murine IL-5R $\alpha$  subunit pre-mRNA with uniformly 2'-MOE-modified ASOs produced skipping of the immediate downstream exon. In addition, hybridization of 2'-MOE ASOs to sequences in the proximity of the 3' splice site of exon 9 of the IL-5R $\alpha$  blocked inclusion of exon 9 and redirected the splicing machinery to the next downstream splice acceptor site (in intron 9). These effects result in the loss of the membrane form mRNA for the IL-5R $\alpha$  chain as well as the superinduc-



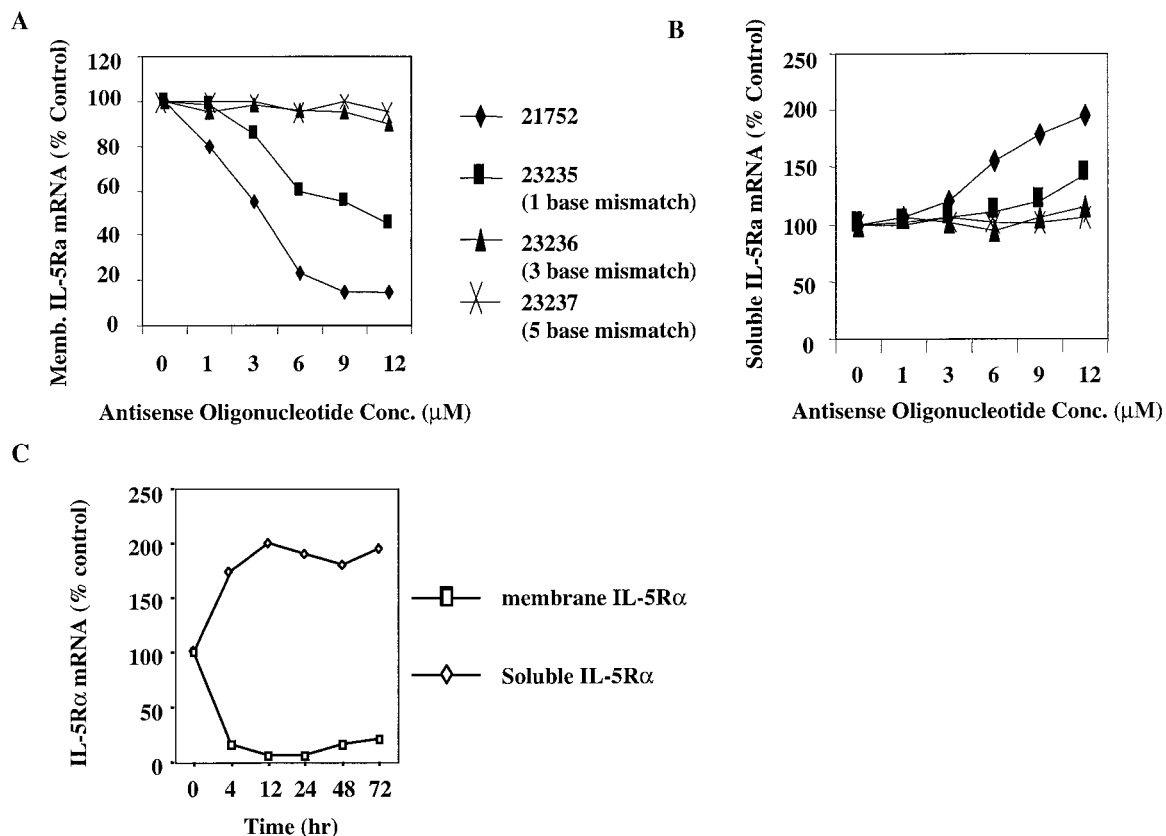
**Fig. 5.** Concomitant inhibition of membrane IL-5R $\alpha$  and superinduction of soluble IL-5R $\alpha$  mRNA by uniformly 2'-MOE-modified ASOs in BCL<sub>1</sub> cells. A, positions of ASOs on the exon 9 sequence and across the intron-exon borders. The bracketed sequences represent exon 9 and available flanking intron sequences. ISIS 21750-21760 are uniformly 2'-MOE-modified ASOs. B, representative RPA image of effect of ISIS 21752 versus ISIS 21758 on membrane (top band) and soluble forms (next lower band) of IL-5R $\alpha$  mRNA 24 h after electroporation into BCL<sub>1</sub> cells. Undigested probes (lane 1), electroporation but no oligonucleotide treatment (lanes 2 and 3), ISIS 21752 (lanes 4 and 5), and ISIS 21758 (lanes 6 and 7) treatment groups are shown. Below the phosphorimage, quantitation of three individual experiments is shown, including S.E.M. These data were derived from analysis of RPA gels 24 h after electroporation with ASOs. The data are normalized to L32 levels. C, quantitation of RPA phosphorimage analysis of effects of uniformly 2'-MOE-modified ASOs depicted in Fig. 2A on membrane (open bars) and soluble (shaded bars) IL-5R $\alpha$  mRNA levels in BCL<sub>1</sub> cells. Data analysis was performed as noted in B. AIC2a,  $\beta$ -subunit of IL-3, IL-5, and GM-CSF receptors.

tion of the soluble form mRNA. These observations potentially extend the therapeutic utility of antisense technology in that oligonucleotides may now be rationally designed to inhibit expression of disease-specific domains in proteins and loss of expression of the whole protein may be avoided. This strategy may be useful for systems in which alternatively spliced transcripts convey opposing function in vivo or where loss of the architectural or adaptor roles of proteins may be detrimental.

That the exon 9 targeted gapmers equally inhibited expression of both IL-5R $\alpha$  isoforms suggests pre-mRNA is the site of hybridization, consistent with earlier studies (Condon and Bennett, 1996). In addition, the ability of uniformly modified 2'-MOE ASOs to almost completely inhibit expression of membrane-encoding IL-5R mRNA while simultaneously increasing levels of transcript that encodes soluble IL-5R $\alpha$  by nearly 100% suggests that these oligonucleotides are redirecting the splicing machinery such that the overall rate of transcription is not affected. The observation that 2'-MOE-modified ASOs hybridizing across or near 3'-splice sites induced skipping of the following exon suggests that the mechanisms of oligonucleotide-mediated modulation of alternative and constitutive splicing may be similar. A potential mechanism of interference of oligonucleotides bordering intron-exon boundaries involves blocking the binding of proteins that regulate splice-site selection, as observed in *Drosophila*

where Sxl binding blocks use of the strong default 3'-splice site in *tra*, thus forcing selection of the weaker distal 3'-splice site (Sosnowski et al., 1989). As a consequence, the resulting processed mRNA would lack a single exon in each case. However, alternatively, hybridization of oligonucleotides to exon sequences may block the function of exonic enhancers (Hertel et al., 1996; Manley and Tacke, 1996; Schaal and Maniatis, 1999) or communication across the exon, as proposed by Berget and colleagues (Robberson et al., 1990; Niwa et al., 1992).

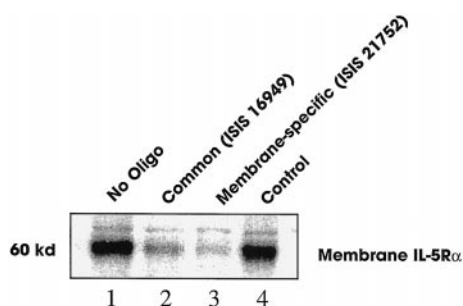
Previous work has shown that 2'-O-methyl ASOs produce inhibition of constitutive splicing of adenovirus pre-mRNAs inserted into luciferase reporter plasmids when weak splice sites are created in the pre-mRNA by mutation (Hodges and Crooke, 1995). In this study, it was found that weak consensus branch point and 3'-intron splice-site sequences were particularly good targets for ASOs. However, because limited intron sequence is known for the mouse IL-5R $\alpha$  chain gene, the pertinent consensus splicing sequences are not readily available. It is tempting to speculate that the unusually large size of intron 8 estimated by polymerase chain reaction (Imamura et al., 1994) and the equal frequency of alternative splicing as opposed to constitutive splicing of exon 9 foretells the existence of a weak splice site at this location. Partial sequencing of intron 8 will be required to address this possibility.



**Fig. 6.** Sequence specificity and kinetics of ISIS 21752-mediated inhibition of membrane IL-5R $\alpha$  mRNA and superinduction of soluble IL-5R $\alpha$  mRNA. Dose-response analysis of membrane IL-5R $\alpha$  mRNA reduction (A) and of soluble IL-5R $\alpha$  mRNA superinduction (B) with ISIS 21752 ( $\blacklozenge$ ) versus its 1 ( $\blacksquare$ ), 3 ( $\blacktriangle$ ), or 5 ( $\times$ ) base mismatch control oligonucleotides. BCL<sub>1</sub> cells were electroporated with either ISIS 21752 or its mismatch controls and RNA harvested and analyzed by RPA 24 h later. Data shown are from phosphorimage analysis of RPA gel and are normalized to L32 levels. C, kinetic analysis of inhibition of membrane ( $\square$ ) IL-5R $\alpha$  mRNA and superinduction of soluble ( $\diamond$ ) IL-5R $\alpha$  mRNA levels. After electroporation of BCL<sub>1</sub> cells with ISIS 21752, RNA samples were prepared at the indicated times and subjected to RPA analysis for IL-5R $\alpha$  isoform measurement. Data shown are from phosphorimage analysis of RPA gel and are normalized to L32 levels.



Kole and colleagues have previously used an ASO approach to correct aberrant splicing caused by intron mutation in thalassemic  $\beta$ -globin mRNA (Sierakowska et al., 1996). In these studies, phosphorothioate 2'-*O*-methyl oligoribonucleotides were targeted to an aberrant 5'-splice site of the pre-mRNA expressed in stably transfected HeLa cells and blocked aberrant splicing, apparently by an occupational mechanism. This strategy is potentially important for treatment of diseases where known mutations are mapped. Additional studies in this system with  $\beta$ -globin constructs and splicing extracts showed that aberrant splicing of mutant  $\beta$ -globin mRNAs could be blocked by antisense targeting within the terminal 25 bases of exon sequence at either the 5' or 3' ends (Dominski and Kole, 1994). Moving the oligonucleotides toward the middle of the exon resulted in a less potent inhibitory effect. Our observations extend the practical applicability of antisense-mediated modulation of splicing demonstrated in these studies by illustrating effects on wild-type, endogenously expressed pre-mRNAs. Recently, in agreement with our data, modulation of *bcl-x* mRNA isoform expression has been observed with 2'-MOE ASOs targeted to regions near a 5'-splice site, by inducing the recognition of a



**Fig. 7.** Inhibition of membrane IL-5R $\alpha$  protein by ISIS 21752. Western blot of Triton X-100 insoluble fraction of whole cell extracts from BCL<sub>1</sub> cells electroporated with 10  $\mu$ M common sequence (ISIS 16949), membrane-specific (ISIS 21752), or control ASOs (10  $\mu$ M each ISIS 21853 and ISIS 21855). ISIS 21853 and 21855 are targeted to sequences uniquely present only in processed soluble receptor isoform mRNA constituted by splicing of exon 8 to exon 10 or to exon 11, respectively. Extracts were prepared 48 h after transfection and probed with antimurine IL-5R $\alpha$ . Only the ~60-kD membrane receptor was detected. The common sequence ASO is targeted against sequence within the 3'-UTR, thus inhibiting all isoforms of IL-5R $\alpha$  mRNA (data not shown). Equal amounts of total protein were loaded in each lane. The data shown are representative of two separate experiments.

**TABLE 1**  
Oligonucleotides used in this study

ISIS No.	Sequence	Descriptor
16949	CATCTGCTTCTGTATTGCCA	common sequence ASO 2'-MOE gapmer
18002	GCCATTCTACCAAGGACTTC	exon 9 2'-MOE gapmer
21750	GACTTCCTTTCCCTTCCTGG	3' intron 8 ss oligo
21752	GCCATTCTACCAAGGACTTC	Exon 9 uniform 2'-MOE
21761	CGATCAGTTTTCTCTCTAA	3' intron 7 ss oligo
21762	TCACCCACATAAATAGGTTG	5' intron 8 ss oligo
21763	GGTCCATAAATGACACCTGA	3' intron 9 ss oligo
21853	TAAATGACACCCACATAAAT	exon 8/10 junction ASO 2'-MOE gapmer
21855	TCGAAGGTTTCCACATAAAT	exon 8/11 junction ASO 2'-MOE gapmer
23235	GCCATTCTATCAAGGACTTC	1 base mismatch/ISIS 21752
23236	GCCATGCTATCAAGCACTTC	3 base mismatch/ISIS 21752
23237	GCTATCCTATCAAGCAGTC	5 base mismatch/ISIS 21752

ss, splice site.

nearby cryptic splice site (Taylor et al., 1999). The observed differences between our study and the  $\beta$ -globin mutant pre-mRNAs suggest that splicing regulation by protein factors and/or small nuclear ribonucleoprotein particles is likely to be complex and perhaps different between in vitro splicing systems and intact cells. Importantly, 3'- and 5'-splice site targeting provides a more general strategy for globally influencing splicing patterns and producing modular transcripts from normal genes.

For IL-5R $\alpha$ , differences in mRNA regulation exist between the mouse and human genes. Although modulating alternative splicing of murine IL-5R $\alpha$  is necessary to specifically target the membrane-bound receptor, in the human, in-frame stop codons in exons 10 and 11 produce soluble forms of the receptor, in the absence of alternative splicing (Tavernier et al., 1991, 1992; Tuypens et al., 1992; Murata et al., 1992). Thus, targeting of exons downstream from exon 10 with RNase H-supporting oligonucleotides may yield human membrane-specific oligonucleotides of potential therapeutic utility. Additionally, in contrast to BCL<sub>1</sub> cells as well as enriched populations of mouse eosinophils harvested from the peritoneum of allergen-challenged mice that express approximately equal levels of mRNA for the membrane and soluble forms of the receptor, human TF-1 cells greatly over-express the soluble form of the receptor mRNA (data not shown). This also has been reported for human eosinophils (Tavernier et al., 1991). Evidence exists that human soluble IL-5R $\alpha$  can inhibit IL-5 signaling responses by binding IL-5 in solution to prevent its interaction with  $\beta$ -chain-associated membrane IL-5R $\alpha$  chains. Thus, a membrane-specific ASO approach may be more therapeutically useful than a small-molecule drug that prevents ligand binding.

In conclusion, this study has shown that ASOs designed to bind with high affinity to 3'-splice sites or flanking exon sequences in pre-mRNA result in specific deletion of individual exons and modulation of transcript isoform expression, without affecting the overall rate of splicing of the pre-mRNA. These splicing effects were observed for the normal murine IL-5R $\alpha$  gene in unmanipulated intact cells, whereas previous work used splicing extracts or gene overexpression. Thus, ASOs are potentially useful for controlling mRNA processing in vivo and should provide novel strategies for creating useful therapeutics that take advantage of our increased understanding of the distinct biology of related proteins that arise from differential splicing.

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