Altered mRNA Splicing and Inhibition of Human E-selectin Expression by an Antisense Oligonucleotide in Human Umbilical Vein Endothelial Cells*

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We have characterized the mechanism of action of an antisense oligodeoxynucleotide (ASO) targeting human endothelial leukocyte adhesion molecule, E-selectin. ISIS 4730, a 20-base ASO designed to be complementary to a region in the 3′- untranslated region (3′-UTR) of human E-selectin, is a potent and specific inhibitor of both mRNA and protein expression in human umbilical vein endothelial cells. Following treatment with ISIS 4730, a lower molecular weight mRNA (3300 bases) species was detected by Northern blot analysis with a corresponding decrease in the mature E-selectin transcript (3875 bases). The ASO-induced low molecular weight mRNA is stable and remains in the nucleus. We demonstrate that ISIS 4730 targets E-selectin pre-mRNA in the nucleus and promotes cleavage of the pre-mRNA at the hybridization site, resulting in prevention of splicing of the last intron. The change in molecular weight of the E-selectin transcript is the result of loss of the 3′-UTR due to ASO-mediated RNA cleavage and retention of the last intron. Cleavage of the E-selectin pre-mRNA appears to be due to endogenous RNase H or a related enzyme activity.

The field of ASO research has grown rapidly over the past few years (1–3). In cell culture-based experiments, most of the work reported has utilized either phosphodiester or phosphorothioate ASO. Phosphorothioate ASO have a sulfur atom substituted for one of the nonbridging oxygen atoms in the phosphate backbone of the DNA. This substitution imparts a greater stability to serum and cellular nucleases (4) and allows use for in vivo applications. The mechanism of action by which ASO may work has been hypothesized to be varied and complex with the target molecule for ASO presumed to be the pre-mRNA or the mature mRNA (5, 6). Potential mechanisms whereby an ASO may inhibit expression of its targeted mRNA include inhibition at the transcriptional or translational levels, as well as specific RNA processing steps (e.g. 5′ capping, polyadenylation, splicing, nuclear export, and degradation of the target RNA by RNases). Also, the mechanism by which ASO may inhibit expression may depend on the cell type being targeted, the particular mRNA being targeted, the target site on the mRNA, and the chemical nature of the ASO.

E-selectin, also known as endothelial leukocyte adhesion molecule-1 (originally ELAM-1), is transiently expressed on endothelial cells by induction with inflammatory mediators such as interleukin-1, TNF-α, or bacterial lipopolysaccharide (7). E-selectin plays a major role in the recruitment of leukocytes to areas of infection or disease. The process by which leukocytes migrate out of the vasculature has been hypothesized to involve at least three steps (8). The first step, involving E-selectin, is transient and results in the rolling of leukocytes along the blood vessel wall. E-selectin has been shown to be transiently expressed in acute inflammatory reactions such as reperfusion injury (9). E-selectin may also serve as a receptor for skin-homing memory T lymphocytes and is expressed at high levels in a variety of chronic inflammatory skin disorders and rheumatoid arthritis (10). Therefore, inhibition of E-selectin expression could be of potential benefit in inflammatory diseases.

We have previously reported on the inhibition of endothelial cell adhesion molecules with ASO (11). We have shown that ASO are specific and potent inhibitors of ICAM-1, vascular cell adhesion molecule-1, and E-selectin expression in HUVEC. One ASO, ISIS 4730, designed to be complementary to an area in the 3′-UTR of the human E-selectin mRNA, displayed a marked reduction in mRNA and protein expression levels. However, reduction of target mRNA was accompanied by a corresponding appearance of a lower molecular weight RNA species that hybridizes to the E-selectin cDNA probe. The size of the novel RNA species was larger than that predicted for a RNase H-mediated cleavage of the E-selectin mRNA. We have now characterized this lower molecular weight transcript and demonstrate that ISIS 4730 targets the E-selectin pre-mRNA, promoting cleavage of the pre-mRNA at the hybridization site. We also demonstrate that ISIS 4730 prevents splicing of the last intron (intron 13) of E-selectin, resulting in an additional 1300 bases of sequence associated with the stable cleavage product.

MATERIALS AND METHODS

Cells and Reagents—HUVEC were purchased from Clonetics (San Diego, CA) and cultivated in endothelial basal media supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Opti-MEM and Lipofectin reagent were purchased from Life Technologies, Inc. Dulbecco’s PBS was purchased from Irvine Scientific (Irvine, CA). Sterile, 12-well plates and Facsflow solution were purchased from Becton Dickinson (Mansfield, MA). Ultrapure formaldehyde was purchased from Polysciences (Warrington, PA). Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN). Fraction V bovine serum albumin was purchased from Sigma. The specific conjugated antibody anti-CD62E-phcoerythrin was purchased from Ancell Corporation (Bayport, MN). The control conjugated antibody, mouse IgG1-fluorescein isothiocyanate was purchased from Pharmingen (San Diego, CA). Catrimox-14 was purchased from Iowa Biotechnology Corp (Oakdale, Minnesota). The control conjugated antibody, mouse IgG1-fluorescein isothiocyanate was purchased from Pharmingen (San Diego, CA). Catrimox-14 was purchased from Iowa Biotechnology Corp (Oakdale, Minnesota).
A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A 1:100 solution of RT-PCR kit was purchased from Stratagene (La Jolla, CA). A cDNA labeling kit, Prime-a-Gene, and RNAsin were purchased from Promega (Madison, WI). The genomic E-selectin clone (12) was a gift from Dr. Tucker Collins, Harvard Medical School, Boston, MA. NAP-5 columns were purchased from Pharmacia (Uppsala, Sweden). The Gene-Clean kit for purifying DNA fragments was purchased from BIo 101 (Vista, CA).

**Oligonucleotide Treatment**—Cells were treated with oligonucleotides as described previously (13, 14). E-selectin expression was induced by adding 5 ng/ml TNF-α to growth medium for 2 h for Northern blot analysis of mRNA.

**Flow Cytometry**—Following oligonucleotide treatment, cells were detached from the plates with D-PBS (without calcium and magnesium) supplemented with 4 mM EDTA (15). Cells were transferred to 12 × 75-mm polystyrene tubes and washed in 2% bovine serum albumin, 0.2% sodium azide in D-PBS at 4 °C. Cells were centrifuged at 200 × g, and the supernatant was decanted. Specific antibody was then added at 1:100 for E-selectin-phycoerythrin, and the control IgG1 was added at 1 μg/ml in 0.1 ml of the above buffer. Antibodies were incubated with the cells for 30 min at 4 °C in the dark, under gentle agitation. Cells were washed again as above and then resuspended in 0.3 ml of FacsFlow buffer with 0.5% formaldehyde. Cells were analyzed on a Becton Dickinson FACScan. Results are expressed as percentage of control expression based upon mean fluorescence intensity, which was calculated as follows: (((CAM expression for oligonucleotide-treated cytokine induced cells) – (basal CAM expression))/ (cytokine-induced CAM expression) – (basal CAM expression)) × 100. Both basal and cytokine-stimulated control cells were pretreated with Lipofectin reagent for 4 h in the absence of oligonucleotides.

**RNA Isolation and Analysis**—Total cellular RNA was isolated either by cellular lysis in 4 M guanidinium isothiocyanate followed by a CsCl gradient (16) or by cellular lysis in Catrimox-14 solution (17). Total cellular RNA was separated on a 0.8 or 1% agarose gel containing 1.1% formaldehyde and visualized by pouring boiling 0.1% SSC, 0.1% SDS solution on the blots and incubating under gentle agitation for 5 min. Blots were reprobed with G3PDH (Clontech, Palo Alto, CA) to confirm equal RNA loading.

**RT-PCR of the Novel mRNA**—RT-PCR was performed on total RNA using a StrataScript RT-PCR kit from Stratagene (La Jolla, CA). Oligonucleotide 1807 (5′-CTTGTGGATCGGAC-GTCTGCT-3′) the 5′ primer and oligonucleotide 1808 (5′-ACAGACCCGAGGAGA-3′) the 3′ primer were used to amplify the novel sequence. The resulting PCR product was sequenced for base confirmation with the Sequenase PCR product sequencing kit from U.S. Biochemical Corp.

**Oligonucleotide Synthesis**—Phosphorothioate oligodeoxyribonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) as described previously (13, 14). Oligonucleotides were analyzed by capillary gel electrophoresis on denaturing gels and judged to be at least 85% full-length material. Following are sequences of ASO used: ISIS 2679, 5′-CTGTGGATCGGAC-GTCTGCT-3′; ISIS 4719, 5′-ACAGACCCGAGGAGA-3′; ISIS 4729, 5′-CTGTTGATCGGAC-GTCTGCT-3′; ISIS 4730, 5′-ACAGACCGAGGAGA-3′; and ISIS 4731, 5′-CTGTTGATCGGAC-GTCTGCT-3′. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad. A Zeta-Probe nylon blotting membrane was purchased from Bio-Rad.

**Northern blot because ISIS 2679 hybridizes to bases 40–59.**

**Reduction of E-selectin mRNA by ASO.** Northern blot analysis was performed with four of the most active E-selectin ASO. HUVEC were treated with 200 nM of the ASO in the presence of Lipofectin reagent for 4 h. Medium was removed and replaced with medium containing 2.5 ng/ml TNF-α for 2 h to induce mRNA. Total cellular RNA was prepared as described under “Materials and Methods.” Blots were hybridized with an E-selectin cDNA probe designed to hybridize to the protein coding region (A) or a G3PDH cDNA probe (B) as described under “Materials and Methods.” The blot was stripped of the E-selectin probe and rehybridized with the G3PDH probe to demonstrate equal loading.

that demonstrated potent inhibitory effects on E-selectin cell surface expression (11) to evaluate their effects on total E-selectin mRNA. Each ASO resulted in a unique mRNA pattern. ISIS 2679 was designed to hybridize to an area in the 5′-UTR of E-selectin mRNA and resulted in a small reduction in the mature mRNA level at the concentration studied (Fig. 1A). Presumably, if ISIS 2679 worked by an RNase H cleavage mechanism, a cleavage product would be difficult to resolve by Northern blot because ISIS 2679 hybridizes to bases 40–59. ISIS 4719 was designed to hybridize at positions 2993–3012 and shows two distinct mRNA bands of approximately 3000 and 3900 bases, consistent with the expected size of a stable RNase H cleavage product. ISIS 4729 was designed to hybridize to positions 2063–2082, and only a reduction in the mature E-selectin mRNA was observed. ISIS 4730 was designed to hybridize at position 2006–2025 of the E-selectin mRNA, and the expected RNase H cleavage products would both be approximately 1900 bases in length. A marked reduction in the mature E-selectin mRNA transcript was observed as well as a transcript of unexpected size (3.3 kilobase pairs) which hybridizes to the E-selectin cDNA probe in the ISIS 4730-treated group. The blot was reprobed with G3PDH to ensure equal loading (Fig. 1B).

To aid in the characterization of the novel mRNA, a series of PCR-amplified DNA probes complementary to different regions of the E-selectin mRNA were prepared (Fig. 2A). Northern blot analyses were performed using total RNA, with separate gels run for each probe to minimize any background contamination from stripping the blot. **Probe 1**, corresponding to the complete protein coding region, hybridized to both the mature and the novel E-selectin mRNA transcript. **Probe 2**, designed to be complementary to the 3′ end of the protein coding region, gave similar results as probe 1 in the ISIS 4730-treated lane. **Probes 3, 4, and 5** designed to hybridize to different areas in the 3′-UTR, all failed to hybridize to the novel E-selectin mRNA, indicating that most, if not all, of the 3′-UTR is missing. However, all probes did hybridize to a band approximately 2000 bases in length which would correspond to the 5′- or 3′-RNase H cleavage products of the mature E-selectin transcript. All five probes hybridized to the TNF-α-induced mature E-selectin mRNA. In contrast to the effects seen with ISIS 4730, treatment of cells with ISIS 4719 and ISIS 4729 resulted in E-selectin mRNA products consistent with an RNase H mechanism of cleavage. **Probes 1, 2, 3, and 4** hybridized to the mature and low

**RESULTS**

Northern blot analysis was performed with four phosphorothioate ASO, ISIS 2679, ISIS 4719, ISIS 4729, and ISIS 4730...
molecular weight transcript for the ISIS 4719-treated cells, whereas, probe 5 hybridized to the mature mRNA and a band of approximately 900 bases in length, which is presumably the 3' cleavage product (Fig. 2B). This pattern of probe hybridization for ISIS 4719 is consistent with an RNase H cleavage mechanism. The mRNA profile for ISIS 4729-treated cells shows a reduction of the mature E-selectin mRNA as well as a low molecular weight mRNA transcript. All five probes hybridized to both the mature and low molecular weight transcripts in the ISIS 4729-treated lanes. This would be consistent with an RNase H cleavage product mechanism because both cleavage fragments should be of approximately equal length and not resolved by this gel system. The control ASO, ISIS 7253, did not alter the mRNA expression pattern for E-selectin mRNA.

Since the ISIS 4730 novel transcript size was inconsistent with its predicted RNase H cleavage pattern on Northern blot, we reasoned that there must be some additional unique sequence present. The hybridization site on the E-selectin mRNA for ISIS 4730 corresponds to the beginning of exon 13. Intron 13, approximately 1300 base pairs in length, is directly upstream from exon 13 (12). A PCR-amplified DNA probe was made that would selectively hybridize to intron 13 (see "Materials and Methods"). The intron 13 probe hybridized to a band of approximately 3 kilobase pairs in length only in the ISIS 4730-treated lane (Fig. 3A) The blot was stripped and hybridized with probe 2, and the characteristic mRNA pattern for each of the groups was seen (Fig. 3B). When the two autoradiographs were overlaid, the bands from probe 2 and the intron 13 probe migrated to the exact location of the low molecular weight mRNA band in the ISIS 4730-treated group. The blot was reprobed with G3PDH to ensure equal loading (Fig. 3C).

RT-PCR analysis on the same RNA used in the Northern blot from Fig. 3 was performed to positively confirm the presence and identity of intron 13 in the low molecular weight mRNA from ISIS 4730-treated cells. Only the ISIS 4730-treated group produced a band of the predicted size, 1370 bases (Fig. 4). The band was gel-purified, sequenced, and found to be exactly the same as intron 13 (12). Therefore, ISIS 4730 appears to mediate cleavage of the E-selectin transcript and prevent splicing of intron 13.

To test whether the cleavage of the E-selectin transcript was due to RNase H, analogs of ISIS 4730 were synthesized, which were uniformly substituted with 2'-fluoro or 2'-O-methyl modifications and are not substrates for RNase H (20, 21). HUVEC...
were treated with ASO as described earlier and cell surface expression was analyzed by flow cytometry. ISIS 4730 inhibits E-selectin expression in a dose-dependent manner, while the 2'-fluoro and 2'-O-methyl analogs showed no effect on E-selectin expression at all doses tested (Fig. 5). Also tested was a 2-base mismatch of ISIS 4730, which did not inhibit E-selectin cell surface expression (data not shown). The 2'-modified analogs also had no effect on E-selectin mRNA (data not shown). Taken together, these data suggest cleavage of the E-selectin transcript by ISIS 4730 is dependent on RNase H or a related enzymatic activity.

The appearance of the induced, novel low molecular weight ISIS4730mRNAtranscript was dependent on the oligonucleotide concentration (Fig. 6). It was first detected at 25 nM and then maximally at 200 nM. The mature mRNA transcript diminishes in quantity, whereas the novel transcript increases in quantity as the dose of ISIS 4730 increases. The quantity of G3PDH does not change with increasing concentrations of ISIS 4730 (Fig. 6).

The kinetics of accumulation of the mature E-selectin and novel low molecular weight mRNA transcript were determined by Northern blot analysis of total RNA. HUVEC were treated with 200 nM of ISIS 4730 and induced with TNF-α for varying lengths of time. The mature E-selectin mRNA transcript was present at 1 h post cytokine induction, peaks at 4 h, and is still detectable at 20 h (Fig. 7). The kinetics of the novel low molecular weight ISIS 4730 mRNA transcript were very similar to the mature E-selectin mRNA results. The relative abundance of each transcript cannot be determined because different probes with different specific activities were used.

As the ISIS 4730-induced transcript contains the entire protein coding region and appears to be stable yet does not translate into protein product, the subcellular localization of the E-selectin transcript was examined to determine if the RNA was translocated to the cytoplasm. ISIS 4730-treated and TNF-α-induced HUVEC were separated into nuclear and cytoplasmic fractions. Total RNA was prepared from each fraction as described previously. Equal amounts of the mature E-selectin mRNA were found in both the cytoplasmic and nuclear fractions. Total RNA was prepared from each fraction as described previously. Equal amounts of the mature E-selectin mRNA were found in both the cytoplasmic and nuclear fractions of the TNF-α-induced HUVEC group at all time points assayed (Fig. 8). In contrast, the majority of E-selectin mRNA in the ISIS 4730-treated group was found in the nuclear fractions at all time points assayed. Three predominant bands are found in the ISIS 4730-treated nuclear fractions: the top band, which corresponds to the mature mRNA, the middle band, and the lowest band, which corresponds to the novel low molecular weight transcript.
which corresponds to the novel intron 13-containing mRNA, and the bottom band, which corresponds to the 5′-RNase H cleavage product of the mature mRNA. The small amount of novel transcript apparent in cytoplasmic fractions could be due to slight contamination during preparation of the cellular fractions. There was no change in G3PDH mRNA distribution in ISIS 4730-treated cells compared to the untreated cells (Fig. 8).

**DISCUSSION**

We have previously described ISIS 4730, an active phosphorothioate ASO that is a potent and selective inhibitor of human E-selectin expression (11). In this study, we have characterized the mechanism of E-selectin inhibition by the ASO, ISIS 4730. ASO have been hypothesized to act at several different stages of RNA maturation and translation, depending on mRNA target site and ASO chemistry. One of the major mechanisms by which ASO are believed to act is through translational arrest by utilizing ASO targeted to the translation initiation codon, although there is no direct evidence that this mechanism occurs in cells (13, 22, 23). A second mechanism of action proposed for ASO is the degradation of the degradation of target mRNA by an RNase H enzymatic activity. Monia et al. (24) have reported the RNase H-dependent cleavage of complementary Ha-ras RNA by 2′-O-methyl chimeric phosphorothioate oligonucleotides. Their study demonstrated the dependence on a minimum number of deoxy residues necessary for the degradation of target RNA in cells, which correlated with RNase H activity in vitro. More recently, Giles et al. (25) have reported direct evidence that RNase H mediates the antisense effects observed intracellularly in cell culture by reverse ligation-PCR and DNA sequencing. Their method requires that the 3′ fragment be stable in cells once it is generated. Our experience has been that this may not always be the case. A third proposed mechanism of action for ASO is the inhibition of RNA splicing. Recently, Hodges and Crooke (26) described studies of a β-globin luciferase plasmid reporter system that explored the utility of ASO for the inhibition of splicing. Also, Dominski and Kole (27) demonstrated the restoration of correct splicing in thalassemic pre-mRNA using ASO in in vitro splicing assays. However, we are unaware of reports that directly demonstrate inhibition of RNA processing intracellularly of an endogenous RNA through the utilization of ASO.

Chiang et al. (13) have previously shown that ASO inhibit ICAM-1 expression by at least two different mechanisms. In that study, it was shown that ISIS 1939, which hybridizes to an area in the 3′-UTR of human ICAM-1, promoted reduction of ICAM-1 mRNA levels, whereas ISIS 1570, which hybridizes to the translation initiation codon, did not reduce ICAM-1 mRNA. Neither ASO changed the transcriptional rate of the ICAM-1 gene, suggesting a post-transcriptional mechanism. 2′-O-Methyl phosphorothioate analogs, which do not support RNase H-mediated cleavage of target mRNA, were utilized to demonstrate that ISIS 1939 was dependent on RNase H for its activity, while ISIS 1570 was not dependent on RNase H for activity. These results suggest that ISIS 1570 is probably a steric blocker of some part of the translation process. Since uniformly 2′-modified fluoro and 2′-O-methoxy analogs of ISIS 4730 were unable to inhibit E-selectin expression or cause formation of a cleavage product, the activity of ISIS 4730 appears to be at least in part due to an RNase H mechanism. However, the possibility that other RNases may cause cleavage of the E-selectin transcript cannot be ruled out.

The hybridization site on the E-selectin mRNA for ISIS 4730 does not appear to be unique, since the splice acceptor and donor sequences of intron 13 and exon 13 agree with the “GT-AG” rule (28) and conform to the consensus sequence proposed by Mount (29) for splicing of introns. The exact sequence of events by which ISIS 4730 exerts its effect remains to be determined. It is possible that ISIS 4730 binds to the pre-mRNA, RNase H cleaves and splicing of the last intron is prevented possibly by loss of the 3′-UTR sequence. This hypothesis would indicate that the cleaved RNA does not inhibit the splicing of the other introns since they are not present. Another possibility is that ISIS 4730 binds to the pre-mRNA transcript and inhibits splicing of intron 13, and once the RNA leaves the spliceosome complex, RNase H cleaves the RNA. To our knowledge, this is the first direct demonstration that ASO have access to endogenous pre-mRNA.

**Fig. 7. Kinetic analysis of the appearance of the novel E-selectin mRNA transcript.** Northern blot analysis of HUVEC treated with Lipofectin reagent in the absence or presence of 200 nm ISIS 4730 for 4 h in serum-free medium. E-selectin expression was induced by treating with TNF-α for 0–20 h followed by total RNA isolation and Northern blot analysis as described under “Materials and Methods.” Data were quantified with a PhosphorImager and is expressed as the percent maximum mRNA expression for the mature and novel E-selectin mRNAs.

**Fig. 8. Subcellular localization of E-selectin and the novel E-selectin mRNA transcripts.** Northern blot analysis of HUVEC treated without ASO or treated with 200 nm ISIS 4730 for 4 h and then induced with TNF-α for 2 h. Nuclear and cytoplasmic fractions were prepared as described under “Materials and Methods.” The full-length E-selectin protein coding region cDNA probe was PCR-labeled with 32P for hybridization and reprobed with G3PDH to ensure equal loading.
Subcellular distribution of the novel ISIS 4730 transcript indicates that it is retained in the nucleus. This observation is in agreement with the “spliceosome retention” model hypothesized by Legrain and Rosbash (30) and Chang and Sharp (31). Their model describes spliceosome assembly and nuclear export as competing processes whereby the spliceosome-pre-mRNA complex is unable to interact with the nuclear pore complex, and export is inhibited. Once the mature mRNA is released from the spliceosome, it can be exported while the introns and spliceosome are retained.

The kinetics of appearance and stability of the mature and novel E-selectin mRNA transcripts appears to be similar. The 3′-UTR of human E-selectin mRNA contains multiple AUUA destabilizing elements and have been shown to confer instability to the corresponding mRNA in many systems (10, 32). The loss of the E-selectin 3′-UTR and its numerous destabilizing sequences after treatment with ISIS 4730 may be involved in the stabilization of the novel E-selectin mRNA transcript. There may also be something unique about intron 13 which stabilizes the transcript.

These data strongly suggest that the primary site of action of ISIS 4730 is in the nucleus. This is further supported by the observation that the delivery of a fluorescein-labeled analog of ISIS 4730 to cells under the conditions used to inhibit E-selectin expression resulted in the accumulation of ASO in the nucleus (data not shown). This result is similar to previous experiments in which we demonstrated that another phosphorothioate ASO delivered to HUVEC in the presence of cationic liposomes accumulate in the nucleus (14). It is unlikely that the cationic lipid has a unique distribution profile for the ASO, since ASO delivered intracellularly by either direct microinjection or electroporation also readily accumulate in the cell nucleus (33, 34).

The observations that we have described here help elucidate the complex mechanisms of action for ASO. We hope that by identifying and characterizing this novel transcript it may aid in designing future splice junction ASO that may possibly change alternatively spliced mRNAs or inhibit splicing altogether. These data may also yield clues to why some ASO targets are more potent than others and may make site selection more predictable in the future.

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