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Correction of Aberrant Splicing of the Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) Gene by Antisense Oligonucleotides*

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The *CFTR* splicing mutation 3849 + 10 kb C → T creates a novel donor site 10 kilobases (kb) into intron 19 of the gene and is one of the more common splicing mutations that causes cystic fibrosis (CF). It has an elevated prevalence among patients with atypically mild disease and normal sweat electrolytes and is especially prominent in Ashkenazi Jews. This class of splicing mutations, reported in several genes, involves novel splice sites activated deep within introns while leaving wild-type splice elements intact. *CFTR* cDNA constructs that modeled the 3849 + 10 kb C → T mutation were expressed in 3T3 mouse fibroblasts and in CFT1 human tracheal and C127 mouse mammary epithelial cells. In all three cell types, aberrant splicing of CFTR pre-mRNA was comparable to that reported *in vivo* in CF patients. Treatment of the cells with 2′-*O*-methyl phosphorothioate oligoribonucleotides antisense toward the aberrant donor and acceptor splice sites or to the retained exon-like sequence, disfavored aberrant splicing and enhanced normal processing of CFTR pre-mRNA. This antisense-mediated correction of splicing was dose- and sequence-dependent and was accompanied by increased production of CFTR protein that was appropriately glycosylated. Antisense-mediated correction of splicing in a mutation-specific context represents a potential gene therapy modality with applicability to many inherited disorders.

Cystic fibrosis (CF)[1] is an inherited disorder characterized by multi-organ involvement and substantial heterogeneity in the presentation of disease (1). At a molecular level, the pathogenesis of CF is attributable in part to over 800 known mutations[2] within the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which interfere with the processing or integrity of the CFTR protein, a cAMP-activated chloride channel (3–6). To the extent that mutations within the *CFTR* gene permit residual chloride channel activity, a milder phenotype typically results.

Approximately 14% of the deleterious mutations known to cause cystic fibrosis interfere with mRNA splicing, a frequency comparable to that reported for other inherited disorders (3, 7).[2] Of the splicing mutations reported, the great majority disrupt either the splice acceptor or splice donor sites that demarcate the 5′ and 3′ ends of each exon, respectively, and drive the exclusion of that exon from the mature transcript.

Splicing may also be derailed by mutations within introns that create novel splice sites, resulting in the inappropriate inclusion of non-coding sequence. This often occurs close to exons, but may also occur deep within introns, creating either a novel donor or acceptor site that, in conjunction with a nearby cryptic splice site of the opposite polarity, defines a novel, aberrant exon that the spliceosome recognizes and includes into the mature message. Several examples of this mutational mechanism have been shown to underlie inherited diseases, such as β-thalassemia (8–12), CF (13, 14), neurofibromatosis type 1 (15), multiple breast tumors (16), dihydropteridine reductase deficiency (17), maple syrup urine disease (18), Alport syndrome (19), ornithine δ-aminotransferase deficiency (20), β-glucuronidase deficiency (21), ataxia-telangiectasia (22), congenital lipoid adrenal hyperplasia (23), and obesity in mice (24, 25). The true prevalence of mutations of this kind is probably underestimated since few laboratories analyze intron sequences far from coding regions.

One of the deep intron mutations that cause CF by the above mechanism, 3849 + 10 kb C → T, is relatively common, with an overall frequency of 1–2% and an elevated prevalence in individuals of Ashkenazi Jewish ancestry (26).[2] It is also the most common mutation among CF patients with normal sweat electrolytes and mild disease (13), as well as among fertile CF men (13, 27). This mutation generates an aberrant 5′ splice site deep in intron 19 of CFTR pre-mRNA and activates a cryptic 3′ splice site 84 nucleotides upstream. Importantly, 3849 + 10 kb C → T and other alleles of this class do not alter wild-type splice sites. With these sequences intact, the mRNA involved can be regarded as retaining the potential for normal splicing, if usage of the aberrant splice sites could be inhibited.

Kole *et al.* (28–30) have previously demonstrated that antisense oligonucleotides or RNAs with an affinity for aberrant 5′ and 3′ splice sites underlying β-thalassemia deterred usage of these elements by the spliceosome in favor of the intact, wild-

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; kb, kilobase pair(s); bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; LTR, long terminal repeat; DMEM, Dulbecco's modified Eagle's medium.

[2] This information can be obtained via the World Wide Web from the Cystic Fibrosis Mutation Data Base (Cystic Fibrosis Gene Analysis Consortium; Hospital for Sick Children, Toronto, Ontario, Canada).

type splice sites, promoting normal splicing patterns and correct expression of the β-globin gene. Here we report the applicability of this approach to the correction of aberrant splicing in the context of CF with enhanced synthesis and processing of CFTR protein.

EXPERIMENTAL PROCEDURES

Vector Construction—CFTR expression vectors were constructed that contain a truncated intron 19 harboring the 3849 + 10 kb C → T locus. The mini-intron was assembled from three PCR fragments of genomic sequence derived from the native *CFTR* intron 19: an *Xho*I-*Hin*dIII fragment encompassing the 3′ end of exon 19, the splice donor site, and the initial sequences of intron 19; a *Hin*dIII-*Sal*I fragment containing the central region of intron 19 including the 3849 + 10 kb C → T and its upstream cryptic acceptor site (this fragment was amplified with or without the 3849 + 10 kb C → T mutation); a *Sal*I-*Bam*HI fragment straddling the intron 19-exon 20 junction that contains the native acceptor splice site and the polypyrimidine tract preceding exon 20. To improve the efficiency of splicing of the constructs in cell culture, the polypyrimidine tract was optimized by the substitution of pyrimidines for three interrupting purines.

Ordered ligation of these fragments into a 415-bp mini-intron was achieved as depicted in Fig. 1*A*. The mini-intron 19 was subsequently incorporated into the LCFSN *CFTR* vector containing the intact coding region of *CFTR* and the Geneticin (G418) resistance genes, controlled by a retroviral LTR promoter (31) using a PCR-based mutagenesis protocol (32, 33). The details of the construction are available on request.

Intron-containing constructs were digested with *Hph*I to detect the presence or absence of the mutation (13) and further by direct sequencing (34). The modified vectors selected for further studies were LCFSN-3849wt (wild-type mini-intron) and LCFSN-3849mut (mutant mini-intron). The wild-type and mutant mini-introns were also inserted in the cytomegalovirus-driven, *CFTR* expression vector, pCF1-CFTR (35) by similar procedures.

Cell Culture—Mouse NIH 3T3 fibroblasts were grown in DMEM-H (high glucose) supplemented with 10% fetal calf serum. The immortalized human tracheal epithelial line, CFT1 (36), was grown in serum-free Ham's F-12 supplemented with factors described elsewhere (37). C127 mouse mammary epithelial cells were grown in DMEM-L (low glucose) supplemented with 10% fetal calf serum. Human T84 colorectal carcinoma cells, which express high levels of *CFTR*, were used for controls and were grown in DMEM/F-12 supplemented with 5% newborn bovine serum. All culture media contained penicillin/streptomycin.

Construction of Stable Cell lines—3T3- or CFT1 cells were stably transfected with either LCFSN-3849wt or LCFSN-3849mut plasmids using LipofectAMINE (Life Technologies, Inc.) and selection with G418 (580 and 150 μg/ml G418, respectively, for the selection and maintenance of 3T3- and CFT1-derived cell lines). 3T3 and CFT1 clonal cell lines containing the wild-type and mutant mini-introns ((3T3-WT8, 3T3-M11, CFT1-WT, CFT1-M15) were selected for further study. Construction of C127-derived mouse mammary epithelial cell lines was accomplished by co-transfection of native or modified pCF1-CFTR expression constructs with the pMEP4 plasmid (Invitrogen), which confers hygromycin resistance. Selection proceeded in 500 μg/ml hygromycin. The C127 cell line stably expressing the ΔF508 *CFTR* mutant was a gift from Genzyme Genetics (Framingham, MA) (38).

Antisense Oligoribonucleotides: Design and Usage—Antisense oligonucleotides were 18 or 19-mer 2′-*O*-methyl oligoribonucleoside phosphorothioates (Hybridon Inc., Milford, MA, and Midland Certified Reagent Co. Woodland, TX) (Table I). They were targeted to either the novel splice donor site, the cryptic splice acceptor site or to a region within the 84-bp aberrant exon that includes an in-frame stop codon (Fig. 1*B*). Anti-β-globin or anti-CFTR oligonucleotides with two mismatched nucleotides were used as negative controls (Table I).

Transfection with Antisense Oligonucleotides (29)—50–100 × 10^3 of 3T3-M11 cells were plated in each well of a 24-well plate 48 h prior to treatment with the oligonucleotide. Antisense oligonucleotides were complexed at room temperature with either 6% LipofectAMINE or 4% DMRIE-C solutions in 200 μl of serum-free Opti-MEM (Life Technologies, Inc.) for 15–30 min. After dilution to 1 ml with Opti-MEM, each mixture was added to cells (pre-washed with Opti-MEM to remove serum) and incubated for 3–5 h. Subsequently, the transfection mixture was replaced with serum containing growth media and cells were harvested 24–48 h after transfection. The antisense oligonucleotide concentrations ranged from 0 to 1.0 μg/ml.

Oligonucleotide treatment of CFT1 cell lines with antisense oligonucleotides was similar and used 5% DMRIE-C in Opti-MEM, 3-h transfections, and harvesting 24 h after transfection. Sodium butyrate, previously shown to boost *CFTR* transgene expression in 3T3-derived cells (39), was added (30 mM) to CFT1 cells for 24 h after transfection. For C127 cell lines, transfection was performed with 2.5% ExGen500 (MBI Fermentas) in Opti-MEM for 3 h. Cells were harvested 24 h after transfection.

RNA Isolation and RT-PCR Analysis—Cells were rinsed once with Hank's balanced salt solution and lysed at room temperature for 5 min with 1 ml of TRI reagent (MRC, Inc.; Cincinnati, OH) per well of transfected cells. Total cellular RNAs were purified according to manufacturer's instructions, and concentrations were measured via spectrophotometry at 260 nm. All RNAs were stored at −20 °C.

RT-PCR was performed using the r*Tth* kit (Perkin-Elmer) with 0.2–0.3 μg of RNA per reaction. The RT reaction was performed in 10 μl for 15 min at 70 °C. [α-^{32}P]dATP was incorporated during the subsequent PCR, performed in 50 μl (95 °C for 3 min, followed by 18–24 cycles of 65 °C for 1 min and 95 °C for 1 min).

Primers (A, 5′-ATCCAGTTCTTCCCAAGAGGC-3′; B, 5′-TCTTCCCAAGAGGCCCACCCTCTG-3′, C, 5′-TCTTCCCAAGAGGCCCACCATTTT-3′; D, 5′-CCAAATGACTGTCAAAGATCTCACAGCA-3′) were designed to detect splicing isoforms arising from processing of the mini-intron as depicted in Figs. 2–7. RT-PCR products (typically 20 μl from the 50-μl RT-PCR sample representing reaction products derived from 120 ng of RNA template) were electrophoresed on 8% polyacrylamide (29:1, acrylamide:bisacrylamide) non-denaturing gels (29), which were dried and autoradiographed for 1–24 h. RT-PCR products derived from T84 RNA template served as a positive control for wild-type splicing. If necessary for further analysis, bands of interest were excised from dried polyacrylamide gels, eluted, re-amplified using primers A and D and sequenced (34).

Western Blots—C127-derived stable cell lines were grown to ~80% confluence on 10-cm plates and transfected with antisense oligonucleotides as described above. For protein isolation, cells were rinsed twice with Hank's balanced salt solution and lysed with 1 ml of 3% SDS, 60 mM Tris, pH 6.8, 1 mM EDTA, 6% sucrose, 100 μg/ml phenylmethylsulfonyl fluoride, 1–2 μg/ml aprotinin, and 1–2 μg/ml leupeptin. Samples were made up to 50 mM dithiothreitol, and Pyronin Y tracking dye was added prior to electrophoresis on 7% SDS-polyacrylamide gels. Proteins were electroblotted to a nitrocellulose membrane and probed with a polyclonal anti-CFTR antibody (α-1468) (40) and a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Detection utilized the ECL kit (Amersham Pharmacia Biotech) and luminography.

RESULTS

Restoration of Correct CFTR Splicing in 3T3-CFTR Cell Line—In order to model the 3849 + 10 kb C → T splicing mutation in cell culture, a 415-bp mini-intron was created by the ordered ligation of three fragments derived from the native 22-kb intron 19 of CFTR (see Fig. 1*A* and "Experimental Procedures"). The construct retained intron 19 native splice sites and approximately 240 bp of sequence bracketing the mutation locus and the cryptic acceptor splice site activated by the mutation. The ligated fragment was accurately inserted at the exon 19-exon 20 junction of the *CFTR* cDNA portion of the LCFSN expression vector (31). The expression of CFTR gene from this vector is driven by retroviral (murine leukemia virus) LTR promoter. The resulting constructs were used to generate 3T3- and CFT1-based cell lines, which constitutively expressed either the wild-type or the mutant mini-intron containing CFTR genes.

Fig. 2 shows RT-PCR analysis of total RNA from a 3T3-based stable cell line (3T3-M11) expressing the 3849 + 10 kb C → T CFTR construct. The cell line did not express detectable levels of correctly spliced CFTR mRNA (Fig. 2*A*, *lanes 2* and *8*) as shown by a lack of the RT-PCR band co-migrating with that of the control CFTR mRNA from T84 cells (Fig. 2*A*, *lane 1*). The correctly spliced fragment was generated after treatment of the 3T3-M11 cells with antisense oligonucleotides (Table I) complexed with cationic lipid carriers and targeted to either the aberrant cryptic acceptor site (Fig. 2*A*, *lanes 2–7*) or the donor site (Fig. 2*A*, *lanes 8–13*). For both oligonucleotides the effects

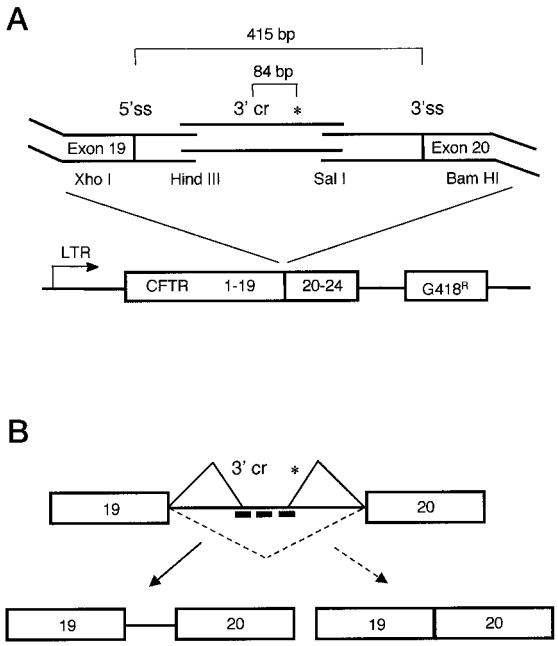

FIG. 1. *A*, modification of LCFSN *CFTR* expression vector to include mini-intron 19. Three fragments of genomic sequence derived from the native or 3849 + 10 kb C → T mutated *CFTR* intron 19 were obtained by PCR. The fragments were cleaved with appropriate restriction enzymes (indicated at the *upper part* of *panel A*), ligated, amplified with tailed primers complementary to exon sequences near the native splice junctions (5′ss, 3′ss, *short vertical lines*), and inserted into LCFSN vector (*lower part* of *panel A*) linearized at the exon 19-exon 20 border with *Pfl*M1 (see "Experimental Procedures" for more details). The position of the mutation locus (*), the cryptic 3′ splice site (3′cr), and the nucleotide lengths of appropriate fragments are indicated. The *open boxes* of the *lower part* of *panel A* represent the CFTR cDNA and Geneticin resistance gene. *LTR*, murine leukemia virus LTR promoter. *B*, splicing pathways of LCFSN-3849mut (mutant mini-intron) pre-mRNA. *Boxes*, exons; *heavy line*, intron. *Thin solid* and *dashed* lines represent aberrant and corrected splicing pathways, respectively. *Heavy short bars*, antisense oligonucleotides targeted to the aberrant 3′ splice site, the stop codon, and the aberrant 5′ splice site, respectively (see "Experimental Procedures" for more details and Table I for oligonucleotide sequences).

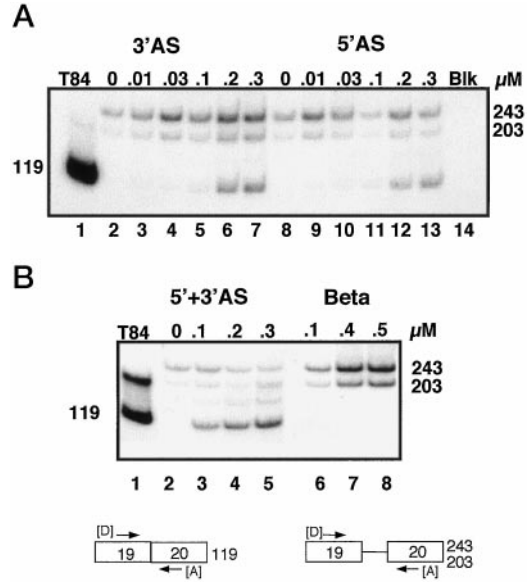

FIG. 2. **Correction of aberrant CFTR splicing in 3T3-M11 cells by antisense oligonucleotides.** *A*, 3T3M11 cells were treated with antisense oligonucleotides (3′AS or 5′AS) targeting the aberrant acceptor and donor splice sites, respectively. Total RNA was isolated and subjected to RT-PCR, and the products were analyzed by polyacrylamide gel electrophoresis. The products and the primers used in RT-PCR are depicted below the panels (see "Experimental Procedures"). *Lane 1*, RNA from T84 cells, positive control for wild-type RNA splicing (*T84*); *lanes 2–7*, correction of aberrant splicing by 3′AS; *lanes 8–13*, correction of aberrant splicing by 5′AS; *lane 14*, water blank. The length, in nucleotides, of RT-PCR products derived from correctly (119) and aberrantly (243, 203) spliced CFTR mRNAs is indicated. Concentrations (μM) of antisense oligonucleotides are shown at the *top*. Unless otherwise indicated similar designations are used in the remaining figures. *B*, *lanes 2–5*, correction of aberrant splicing in 3T3-M11 cells in response to dual antisense oligonucleotide treatment targeting both the aberrant splice donor or cryptic splice acceptor sites; *lanes 6–8*, absence of splicing correction upon treatment with antisense oligonucleotide targeting the β-globin, IVS2–654 aberrant donor site (29).

were dose-dependent. Dose-dependent correction of CFTR pre-mRNA splicing was also observed when both 3′ and 5′ splice site targeted antisense oligonucleotides were combined and used for cell treatment (Fig. 2*B*, *lanes 2–5*). Lack of correctly spliced *CFTR* mRNA in cells treated with an oligonucleotide antisense to the human thalassemic β-globin IVS2–654 aberrant donor site (29), which has no affinity for the CFTR transgene's aberrant splice sites, shows sequence specificity of the effects (Fig. 2*B*, *lanes 6–8*). These results indicate that the antisense oligonucleotides prevented utilization of the aberrant splice sites by the spliceosome and forced it to reutilize the existing correct splice sites, thereby restoring correct splicing of CFTR pre-mRNA.

Characterization of RT-PCR Products; Identification of a Cryptic 5′ Splice Site in Intron 19 of CFTR Gene—RT-PCR of total RNA expressed in 3T3-M11 cell line should in principle produce a single band of 203 bp representing aberrantly spliced 3849 + 10 kb C → T CFTR mRNA (Fig. 2*A*, *primers D* and *A*). After correction of splicing with antisense oligonucleotides, a 119-bp band representing correctly spliced mRNA should also appear. However, in oligonucleotide-treated cells, an array of bands was generated, complicating identification of aberrant products. Thus, we have designed a series of primers, diagrammed in Fig. 3, intended to amplify either all CFTR mRNAs, only correctly spliced mRNAs, or only aberrant mRNAs.

RT-PCR (*primers D–A*, Fig. 3) of RNA from oligonucleotide-treated cells (the same RNA as analyzed in Fig. 2*A*, *lane 13*) detected both the correctly spliced CFTR mRNA that co-migrates with T84-derived mature splicing products (Fig. 3, *lane 1*) and additional species, which presumably included the aberrantly spliced mRNAs (Fig. 3, *lane 2*). The wild-type-specific primers, D–B, amplified only the correctly spliced mRNA, as expected (Fig. 3, *lane 4*), while the aberrant specific primers, D–C, amplified a doublet of bands migrating, at ~240 and ~200 bp, respectively, the smaller of which closely approximated the 196-bp fragment detected *in vivo* (13) (Fig. 3, *lane 5*). The nature of the heavier of the two fragments was not immediately known.

To clarify the identity of the products arising from splicing of the *CFTR* transgenes, RT-PCR generated bands were re-amplified with primers A and D and sequenced. The fragment that co-migrated with the control for wild-type splicing was confirmed to be the product of correct splicing, whereas the smaller of the two heavy bands included the 84-bp insertion reported *in vivo* (13) and therefore represented the expected aberrantly spliced CFTR mRNA. The heavier fragment with an effective mobility of ~240 bp contained, in addition to the sequences found in the smaller aberrant fragment, the first 40 bp of intron 19 inserted between the 3′ end of exon 19 and the 5′ end of the 84-bp aberrant insertion. Analysis of the sequence at the 5′ region of native intron 19 of CFTR gene showed that a previously unrecognized cryptic splice donor site (CA|GTAAGT) resides at position 3849 + 41. Thus, in the 3T3-M11 cell line, the mini-intron undergoes two aberrant splicing events, inclusion

TABLE I
Sequences of antisense oligonucleotides

AS, antisense. Asterisks (*) indicate mismatched bases relative to target sequences. Underlined bases mark the location of the in-frame ochre stop codon within the 84-bp aberrant insert. β-Globin IVS2–654 antisense oligonucleotide as reported (29).

5′AS	Novel donor site	5′-GUCUUACUCACCAUUUUA-3′
5′mis	Mismatched control for 5′AS	5′-GUCUUCCUCACCCUUUUA-3′
3′AS	Cryptic acceptor site	5′-CAAGUCAACUGAAAUUUAG-3′
3′mis	Mismatched control for 3′AS	5′-CAAGUCCACUGAACUUUAG-3′
Anti-stop	In-frame stop codon	5′-CUUGUAAUUAUUUUUACAU-3′
β-Globin IVS2–654 (beta)	β-Globin novel donor site	5′-GCUAUUACCUUAACCCAG-3′

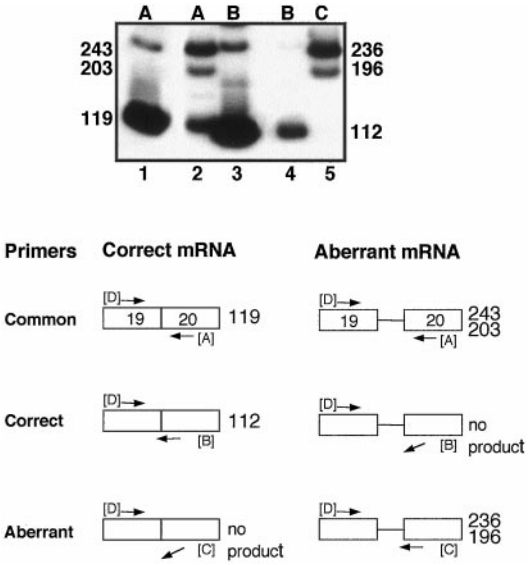

FIG. 3. **Differential RT-PCR analysis identifies wild-type and aberrant CFTR splicing products.** *Lanes 1* and *3*, T84 RNA; *lanes 2*, *4*, and *5*, RNA from 3T3-M11 cells treated with 0.3 μM 5′ AS (same RNA as in *lane 13*, Fig. 2*A*). Primers D and A (*lanes* marked *A*), all *CFTR* mRNA isoforms are amplified. Primers D and B (*lanes B*), RT-PCR specific for correctly spliced CFTR mRNA. Primers D and C (*lane C*), aberrant-specific RT-PCR. The length, in nucleotides, of the resulting RT-PCR products is indicated.

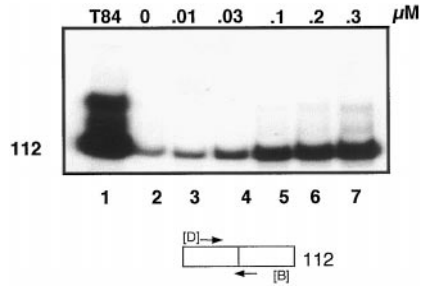

FIG. 4. **Dose responsiveness of antisense-mediated correction of aberrant splicing in 3T3-M11 cells demonstrated with wild-type specific RT-PCR.** *Lanes 2–7*, correction of aberrant splicing in 3T3-M11 cells in response to antisense treatment with oligonucleotide 5′AS, complementary to the aberrant 5′ donor splice site. Same RNAs as in *lanes 8–13* of Fig. 2*A*.

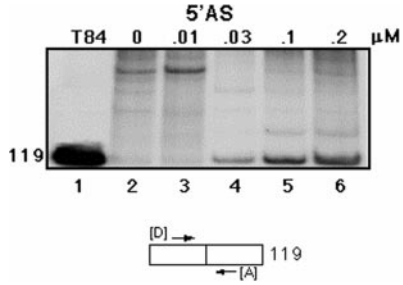

FIG. 5. **Antisense-mediated correction of aberrant splicing in the CFT1-M15 cell line.** *Lanes 2–6*, RNA from cells treated with oligonucleotide 5′AS, complementary to the aberrant 5′ donor splice site.

of the 84 nucleotide "exon" and utilization of a cryptic 5′ splice, resulting in a 243-bp splice isoform.

The products of correct splicing generated in 3T3-M11 cell line by antisense oligonucleotide treatment were additionally identified by RT-PCR analysis with primers D–B (Fig. 4, *lanes 2–7*). The RNAs analyzed in this figure are the same as those in Fig. 2*A* (*lanes 8–13*). A single prominent band co-migrating with the RT-PCR product of T84-derived native CFTR mRNA (Fig. 4, *lane 1*) represents correctly spliced CFTR mRNA and shows with great clarity the dose response of CFTR splicing correction to antisense treatment.

Correction of CFTR Splicing in CFT1 Cells—To more closely approximate the cells that would constitute actual targets if antisense oligonucleotides were used for treatment of cystic fibrosis, the CFT1-based cell lines carrying the modified CFTR transgenes, LCFSN-3849wt or LCFSN-3849mut, were developed. The CFT1 cells, a human papilloma virus-immortalized human tracheal epithelial cell line derived from a CF patient, have retained their differentiated phenotype yet lost the endogenous *CFTR* expression, eliminating a background that might otherwise have obscured detection of antisense-mediated effects (36).

Similar to 3T3-M11 cells, the monoclonal CFT1-M15 cell line exhibited the dose- and sequence-dependent correction of modified CFTR pre-mRNA splicing by antisense oligonucleotide targeted to the 3849 + 10 kb C → T donor splice site (Fig. 5, *lanes 2–6*). The CFT1-M15 line differed from the 3T3-M11 counterpart in that the pair of bands regarded as products of aberrant splicing was either absent or present at much lower levels. It seemed possible that in these cells the aberrantly spliced CFTR mRNA was much less stable than the correctly spliced one because the ochre stop codon within the aberrant exon provided a recognition element for the nonsense-mediated RNA decay machinery (41). Thus, an antisense oligonucleotide targeted to the sequence surrounding the premature stop codon was designed to test if blocking this codon might promote appearance of the aberrant bands. An alternative possibility that correct splicing might be promoted by an antisense oligonucleotide that prevents binding of the splicing factors to the 84 nucleotides of the aberrant exon, thereby interfering with the definition of the exon by the splicing machinery (42), was also considered.

Although the "anti-stop" oligonucleotide showed no utility for increasing the stability of aberrant transcripts, it did promote correct splicing as efficiently as the antisense oligonucleotides targeted to aberrant splice sites, *i.e.* equivalent concentrations (0.3 μM) of the 5′ AS, 3′AS, or anti-stop oligonucleotides were comparably effective (Fig. 6, *lanes 3–5*). Interestingly, dual (or even triple) treatment with all possible AS oligonucleotide combinations appeared more effective than single oligonucleotide treatment, suggesting additive or synergistic effects among the

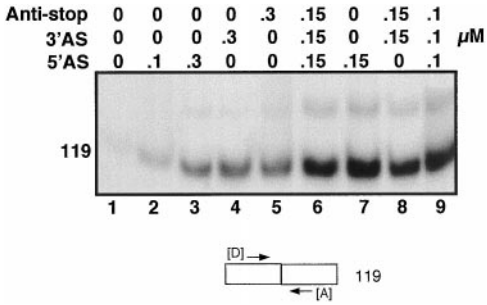

FIG. 6. **Increased correction of CFTR pre-mRNA splicing in CFT1-M15 cells by combined antisense oligonucleotides.** *Lanes 1–9*, CFT1-M15 cells treated with different combinations of antisense oligonucleotides (described above the *lanes*).

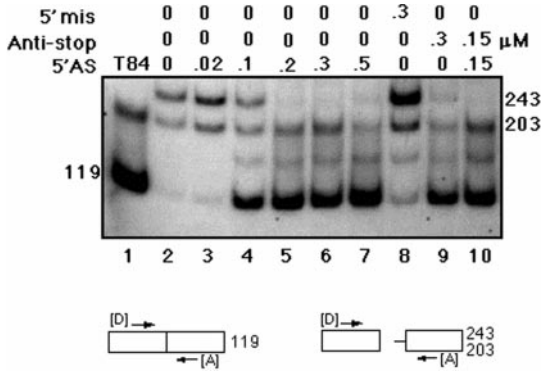

FIG. 7. **Antisense-mediated correction of aberrant splicing in C127-M7 cells.** The cells were treated with the following: *lanes 2–7*, 5′AS oligonucleotide; *lane 8*, 0.3 μM mismatched 5′ AS (negative control); *lane 9*, 0.3 μM anti-stop AS; *lane 10*, 0.15 μM each 5′AS and anti-stop oligonucleotide.

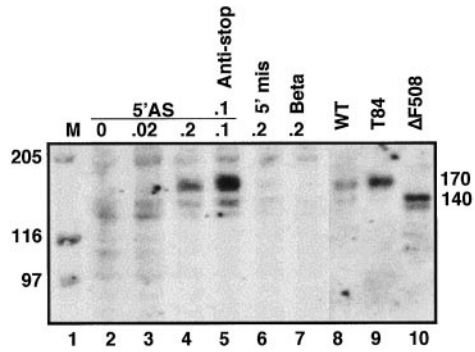

FIG. 8. **Detection in C127-M7 cells of appropriately glycosylated CFTR protein generated by antisense oligonucleotides.** Total cellular protein was analyzed by immunoblotting and detection with a polyclonal anti-CFTR antibody (see "Experimental Procedures" for details). *Lane 1*, *M*, protein size markers (molecular mass in kDa indicated on the *left*). *Lanes 2–4*, C127-M7 cells treated with 0, 0.02, and 0.2 μM 5′ AS, respectively. *Lane 5*, treatment with 0.1 μM each, 5′ AS oligonucleotide targeting aberrant 5′ site and the in-frame stop codon oligonucleotide (anti-stop). *Lanes 6* and *7*, negative controls with 0.2 μM mismatched 5′AS and β-globin IVS2–654 (beta) oligonucleotides, respectively. *Lane 8*, C127 cells transfected with pCF1-CFTRwt construct. *Lane 9*, T84 cells (positive control). *Lane 10*, C127 cells expressing pBPV-CFTR-ΔF508 (38).

oligonucleotides (Fig. 6, *lanes 6–9*).

Restoration of Correct CFTR Gene Expression in C127 Cells—Although expression of aberrant CFTR mRNA and correction of splicing of CFTR pre-mRNA were clearly detectable in 3T3 and CFT1 cell lines, we were unable to detect in oligonucleotide-treated cells the expected correct CFTR protein. Assuming that this was due to a low level of expression of the transgenes in both cell lines, we have explored another cell line-vector combination as a means of attaining sufficiently high levels of *CFTR* expression.

C127 mouse mammary epithelial cells divide rapidly and strongly express CFTR protein when transfected with the pCF1-CFTR construct, in which expression of CFTR cDNA is driven by a strong immediate early cytomegalovirus promoter (35, 40). Transfection of these cells with either native pCF1-CFTR (no intervening introns) or its modified counterpart harboring the wild-type mini-intron 19 (pCF1-CFTRwt) resulted in one prominent RT-PCR product representing mature *CFTR* transcripts (data not shown). A stable cell line (C127-M7) harboring the pCF1-CFTRmut construct (with mutated mini-intron 19) expressed the aberrant CFTR mRNA with greater efficiency than either the 3T3- or CFT1-derived cell lines and produced minimal levels of correct mRNA in the absence of antisense oligonucleotides (Fig. 7, *lane 2*).

The C127-M7 cells were transfected with varying concentrations (0–0.5 μM) of the 5′ AS or anti-stop antisense oligonucleotides using ExGen500 as delivery agent. As shown with the previous cell lines, C127-M7 cells produced correctly spliced *CFTR* mRNA in a dose-responsive manner when treated with the oligonucleotides individually (Fig. 7, *lanes 2–7* and *9*) or in combination (Fig. 7, *lane 10*). Additionally, treatment with a mismatched oligonucleotide, with partial homology for the aberrant donor site, was much less effective at promoting correct splicing, confirming sequence specificity of the effect (Fig. 7, *lane 8*). Identical results were attained using a mismatched oligonucleotide with partial homology for the cryptic splice acceptor site (data not shown).

Expression of the aberrant transgene in C127-M7 cells as well as its responsiveness to antisense treatment were sufficiently robust to assess the synthesis of CFTR protein in oligonucleotide-treated cells by Western blot analysis (see "Experimental Procedures"). Fig. 8 demonstrates an increase in CFTR protein production with increasing dose of antisense oligonucleotides targeted to the aberrant 5′ splice site (Fig. 8, *lanes 2–4*). As with RT-PCR analysis, it is apparent that the treatment with a mixture of two individually effective antisense oligonucleotides, 5′ AS and anti-stop, is superior, on an equimolar basis, to single oligonucleotide treatment (Fig. 8, *lane 5*). As expected, neither the mismatched oligonucleotide, nor antisense oligonucleotide targeted to the human β-globin IVS2–654 splice site were effective at promoting translation of CFTR protein (Fig. 8, *lanes 6* and *7*, respectively). C127 parental cells expressing modified pCF1-CFTR harboring a wild-type mini-intron (pCF1-CFTRwt) were also shown to permit CFTR protein formation (Fig. 8, *lane 8*).

The CFTR protein generated in oligonucleotide-treated cells co-migrated on the gel with CFTR expressed in C127 cells harboring the pCF1-CFTRwt construct or in T84 cells (Fig. 8, *lanes 8* and *9*, respectively). The migration of CFTR protein derived from a C127 cell line expressing the common ΔF508 CFTR mutant was faster (Fig. 8, *lane 10*). This result indicates that, in contrast to ΔF508 CFTR, glycosylation of which is known to be defective (43), the CFTR protein generated by antisense oligonucleotides is wild-type and fully glycosylated.

DISCUSSION

3849 + 10 kb C → T is a member of a class of splicing mutations that, rather than disrupting existing splice sites, create novel ones that are erroneously recognized by the splicing machinery (8–25). Usage of such splice sites results in the abnormal inclusion of intron sequences in the mature transcripts with a wide range of clinical consequences. The exon-shuffling theory (44) proposes that introns serve as reservoirs of new coding sequence and evolutionary potential and mutations like 3849 + 10 kb C → T are mechanistically compatible

with this theory. From the standpoint of immediate clinical consequences, however, molecular derangements of this class must be detrimental in the vast majority of individuals.

The likelihood that an RNA sequence will be recognized by the spliceosome depends chiefly on how well it approximates the consensus splice site sequence. The relation between the consensus sequence and a given splice site may be expressed numerically using the scoring system of Shapiro and Senapathy (45). Relative to the consensus splice site score of 100, 3849 + 10 kb C → T creates a novel donor site with a score of 88.9; the score of the non-mutated sequence at this site is 70.6. By comparison, the native intron 19 donor and acceptor splice sites have scores of 83.2 and 86.7, respectively. This analysis suggests that the novel splice site should be preferred over the native ones by the splicing machinery. Moreover, and probably more importantly, the novel splice site in conjunction with the cryptic splice site located 84 nucleotides upstream in the intron creates an exon-like sequence. The exon definition mechanism predicts that such sequence should be efficiently included in the spliced mRNA (42). Interestingly, despite the high score of the novel donor splice site and creation of the aberrant exon, the impediment to normal splicing is not absolute. *In vivo*, but not *in vitro*, as shown by our results, approximately 8% of the *CFTR* transcripts are normal length (13). This low level of wild-type mRNAs is thought to account for the mild, if variable, phenotypes in patients with the 3849 + 10 kb C → T mutation (13, 27, 46, 47). The retention of wild-type splice sites on 3849 + 10 kb C → T alleles not only permits low levels of normal splicing but also suggested a mechanism by which wild-type splicing could be restored. If usage of the aberrant splicing elements were prevented by antisense oligonucleotides, it seemed likely that the spliceosome will revert to normal utilization of the native splice elements.

Using two different CFTR cDNA expression vectors, LCFSN and pCF1-CFTR, in three different cell lines (3T3, CFT1, and C127), we have demonstrated that antisense oligonucleotides complementary to either the aberrant donor or the cryptic acceptor splice sites promoted the formation of correctly spliced *CFTR* mRNA. This corrective effect was specific for the oligonucleotides with full complementarity to the 18 bp of sequence bracketing each novel splice site. Oligonucleotides with two nucleotide mismatches and an oligonucleotide of wholly unrelated sequence showed no efficacy for the correction of splicing.

The correction of aberrant splicing in multiple cellular contexts speaks to the potential broad applicability of this therapeutic modality. This approach has been also shown to be effective in correction of splicing of human thalassemic β-globin pre-mRNAs (28–30) and in modification of splicing of dystrophin genes (48, 49).

Patients with 3849 + 10 kb C → T reportedly produce one major aberrant mRNA isoform reflecting the inclusion of 84 bp of intron sequence (13). RT-PCR analysis of splicing in the 3T3- and C127-derived mutant cell lines, however, identified two bands associated with aberrant processing. One represented the expected 84-bp insertion, while the second band incorporated the first 40 bp of intron 19 due to splicing at a cryptic splice donor site (CA|GTAAGT) at position 3849 + 41, which, surprisingly, is present but does not appear to be active, in native intron 19 sequence. This site has a Shapiro-Senapathy score of 79.4 in primates and 80.1 in rodents (45) and, if activated, should disrupt the reading frame of the mature message. It is possible that the novel sequence context within the mini-intron may be responsible for its use and/or that the heterologous expression of the constructs in murine cells that do not normally express *CFTR* gave rise, *in vitro*, to this unexpected splicing pathway. However, since this splice site is present in the native *CFTR* intron 19, one must consider that its activation may occur in some patients or in specific tissue contexts. If this is true, the level of usage of this cryptic donor site may contribute to variable disease severity in patients with identical mutational genotypes of the *CFTR* gene.

The anti-stop oligonucleotide that anneals to the vicinity of the ochre stop codon in the aberrant exon promoted wild-type splicing. This result, which was equally apparent in both CFT1- and C127-derived mutant cells, should be viewed in light of the exon definition model that postulates interaction between spliceosome components bridging an exon (42). An oligonucleotide that sterically inhibits this bridging would impair recognition of the exon as sequence to be retained in the mature message. Blocking of the premature stop codon itself was probably not pertinent to this effect.

Superior correction of splicing was seen when two rather than one antisense oligonucleotide were transfected in concert, *e.g.* targeting the 5′ and 3′ splice sites simultaneously. Presumably one oligonucleotide corrects splicing among those transcripts left untouched by the other oligonucleotide. A rigorously quantitative assessment will be required to determine if the effect is synergistic or merely additive.

It is logical to assume *a priori* that correction of splicing would be accompanied by increased production of normal protein. The ribosome should translate correctly processed *CFTR* mRNAs equally without regard to how they came to be correctly spliced. C127 cells stably transfected with the aberrant pCF1-CFTR transgene produced CFTR protein upon treatment with the same oligonucleotides shown to correct splicing. The increase in detectable protein was both sequence- and dose-dependent, with dual oligonucleotide treatment associated with the highest level of protein. Furthermore, we note that the CFTR protein formed in response to antisense oligonucleotide treatment was appropriately glycosylated, suggesting that CFTR polypeptide processing beyond simple translation has also occurred, presumably leading to cell surface expression and chloride conductance.

The potential for antisense-mediated approaches to achieve utility in the clinical arena will depend on several factors. Splicing mutations that evoke aberrant inclusion of intron sequence are currently regarded as infrequent. Although several mutations that fit this category have been reported (8–25), there is likely a bias against their identification. Typically, mutation screening targets coding regions and adjacent splice sites, whereas more cumbersome RNA-based analyses are best suited to discern deep intron mutations. The mutations identified thus far may represent the tip of the iceberg.

Each of the examples cited (8–25), including 3849 + 10 kb C → T, involve a novel splice site in proximity to a cryptic splice site of the opposite polarity. In principle, these can occur at anyplace within an intron. In contrast, many other intronic mutations create splice donor and acceptor sites proximal to exons, leading to the inclusion of intron sequences adjacent to those exons into the mature message (50–57). As with 3849 + 10 kb C → T, wild-type splicing elements remain intact, making these aberrant alleles potential substrates for antisense-mediated correction as well, as long as no steric hindrances with the spliceosome occur. This approach has been tested *in vitro* in the context of thalassemic mutations of human β-globin gene (28, 58).

2′-*O*-Methyl oligoribonucleoside phosphorothioates as antisense oligonucleotides have high affinity for their target sequences, are markedly resistant to intracellular degradation, and, when complexed to their target, will not promote RNase H-mediated cleavage of targeted RNA (59). With such obvious technical utility, it is important that neither the oligonucleo-

tides nor the delivery vehicle is unduly detrimental to the treated cells. Strong antisense-mediated correction has been demonstrated without a dramatic impact on cell morphology or viability. Furthermore, an argument that antisense oligonucleotides might have unforeseen, nonspecific effects on splicing and, consequently, the expression of a multitude of other genes is not supported by gross assessment of cell viability, morphology, and growth rates, all of which appear normal. In view of these promising results, further experimentation and optimization should encompass *ex vivo* or *in vivo* studies in animal models.

The potential for antisense pharmaceuticals to treat human disease depends upon the safe delivery of oligonucleotides at therapeutic doses to appropriate tissues. Many animal studies have demonstrated efficacious delivery of oligonucleotides through systemic treatment (reviewed in Ref. 60), and this approach might prove equally valid for treatment of CF. Alternatively, aerosol delivery of the antisense oligonucleotides directly into CF lungs may efficiently target the impaired tissues. Importantly, treatment with antisense oligonucleotide aerosols markedly reduced allergic response in lungs of rabbits modeling adenosine-mediated hyper-responsiveness (61). The efficient, low dose delivery of potentially therapeutic antisense appeared to be facilitated by weakly cationic lung surfactants, which are known to recycle between type II pneumocytes and the air-liquid interface in the lung (2).

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