HUMAN MUTATION 1:380–387 (1992)

RESEARCH ARTICLE

Characterization of an Intron 12 Splice Donor Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Gene

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Communicated by David Ginsburg

Cystic fibrosis, the most common lethal genetic disease in the white population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Analysis of DNA from a pancreatic insufficient patient by chemical mismatch cleavage and subsequent DNA sequencing led to the identification of a potential splice mutation in the CFTR gene. A transition of the invariant guanosine to adenosine (1898+1G>A) was found at the splice donor site of intron 12. To determine the effect of this mutation on the patient's CFTR transcripts, RNA from the nasal epithelium was reverse transcribed and amplified by the polymerase chain reaction (RT-PCR). Direct sequencing of the PCR products revealed that the transcript from the chromosome with the 1898+1G>A mutation had skipped exon 12 entirely, resulting in a joining of exons 11 and 13. Deletion of exon 12 results in the removal of a highly conserved region which encodes the Walker B consensus sequence of the first nucleotide-binding fold of CFTR.

KEY WORDS: Cystic fibrosis transmembrane conductance regulator, Mutation, RNA splicing

INTRODUCTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the white population, affecting approximately 1 in 2,500 live births (Boat et al., 1989). The disease is characterized by abnormalities in electrolyte transport including defective cAMP regulation of chloride channels in epithelial cells (Quinton, 1990; Welsh, 1990). The gene implicated in CF, the cystic fibrosis transmembrane conductance regulator (CFTR) gene, has been cloned (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989) and the predicted protein product is similar to the family of ATP-binding transport proteins (Ames et al., 1990; Hyde et al., 1990). The structure consists of a repeated motif of six putative membrane spanning segments followed by a nucleotide binding fold (NBF). Separating the two halves of the molecule is a highly charged regulatory (R) domain with multiple phosphorylation sites (Cheng et al., 1991).

Expression of CFTR in CF epithelial cells corrects the chloride transport defect (Drumm et al., 1990; Rich et al., 1990), and expression of CFTR in a variety of heterologous cells is associated with the appearance of cAMP-regulated chloride conductance (reviewed by Collins, 1992). Purified CFTR protein reconstituted in lipid bilayers also forms chloride channels (Bear et al., 1992). These data suggest that CFTR itself is able to function as a cAMP-regulated chloride channel.

The most common mutation responsible for CF is a deletion of the phenylalanine at position 508 of CFTR (ΔF508). This mutation accounts for approximately 70% of CF chromosomes worldwide (Cystic Fibrosis Genetic Analysis Consortium, 1990). In the remaining 30% of CF chromosomes, over 170 putative mutations have been identified, including missense mutations, nonsense mutations, deletions, insertions as well as potential

Received July 22, 1992; accepted August 24, 1992.

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splice mutations (Tsui et al., 1992). Identification and characterization of putative mutations provide insight into the function of CFTR. We have used chemical mismatch cleavage and subsequent DNA sequencing to identify a splice mutation at the 5′ end of the intron following CFTR exon 12. CFTR mRNA transcribed from this allele was found to skip exon 12 entirely.

MATERIALS AND METHODS

Patient Description

Two unrelated CF patients were identified as having ΔF508/1898+1G>A mutations from DNA analysis, as discussed below. Both patients were previously diagnosed as having CF on the basis of clinical findings of typical pulmonary and gastrointestinal disease, together with abnormal values of sweat chloride/sodium test.

Patient "A" was a 39-year-old white male. His height was 169 cm (10% for age) and weight was 51 mg (<5% for age). Tests of pulmonary function revealed FVC of 2.55 L (59% of predicted) and FEV1 of 1.29 L (36%). He was pancreatic insufficient. He had a history of liver disease and required splenorenal shunt for portal hypertension at age 14 years.

Patient "B" was a 9-year-old white female. Her height was 124 cm (10–25% for age), and weight was 26.4 kg (25–50% for age). Tests of pulmonary function revealed FVC of 1.23 L (93%) and FEV1 of 1.04 L (86%). She was pancreatic insufficient.

Detection of Mutation by Chemical Mismatch Cleavage and DNA Sequencing

CFTR exon 12, including more than 100 bp of each flanking intron sequence, was amplified from patient DNA by standard methods (Sambrook et al., 1989). The primers and conditions used were those of Zielinski (1991), except that the primers were modified by the addition of a *Bam*HI restriction site as follows: 5′-ATACGGATCCGTGAATCGATGTGGTGACCA-3′ and 5′-ATACGGATCCCTGGTTTAGCATGAGGCGGT-3′. Chemical mismatch cleavage was performed as described (Strong et al., 1991) based on published methods (Cotton et al., 1988; Grompe et al., 1989). Heteroduplexes were formed between patient and radioactively labeled wild-type DNA by heating the mixture to boiling and slowly cooling to room temperature. Treatment with hydroxylamine and osmium tetroxide modified mismatched nucleotides in the heteroduplexes, which were then cleaved with piperidine. The cleavage products were resolved by electrophoresis on a 6% acrylamide gel and visualized by autoradiography.

DNA determined to contain a mutation based on the chemical mismatch cleavage studies was amplified by PCR, gel purified, digested with *Bam*HI, and subcloned into M13mp18 DNA. Single-stranded DNA was isolated and sequenced by the dideoxy chain termination method using a Sequenase Kit (U.S. Biochemical Corp.)

Allele Specific Oligonucleotide Screening

Oligonucleotides were generated corresponding to the normal (5′-TTTGAAAGGTATGTTCT-3′) and mutant (5′-TTTGAAAGATATGTTCT-3′) sequences. Amplified exon 12 DNA from CF patients and normal individuals was transferred to nylon membranes and hybridized to kinased oligonucleotides as described (Strong et al., 1991), except that the hybridization and washes were done at 39°C.

Analysis of CFTR mRNA from Nasal Epithelium

Nasal tissue was obtained from the patient by nasal scrape using a Rhino-Probe (Synbiotics Corp, San Diego, CA). The sample was placed in phosphate buffered saline and total RNA was isolated by the RNazol B method (Cinna/Biotecs Laboratories, Friendswood, TX). Approximately 1 μg of RNA was reverse transcribed using 1 μg of random hexamers as reverse transcription primers in a 50 μl reaction which contained 500 μM dNTPs, 50 units of RNasin (Promega), 200 units of MMLV-RT (BRL), and a buffer of 50 mM Tris (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, and 10mM DTT. One-fifth of the reverse transcription reaction was used in a 50 μl PCR reaction and the buffer and dNTPs concentrations were adjusted to that of a standard PCR reaction (Sambrook et al., 1989); 250 ng of each primer was included in the reaction. The 5′ primer (5′-CACGGATCCAACCACCATCTCATTCTGCATTGTTCTG-3′) was located in exon 7, and the 3′ primer (5′-CACCTGCAGGTTCAGGAGACAGGAGCATCTCCTTCTAATG-3′) was in exon 13. Reaction conditions were 35 cycles of 1 min at 94°C and 2 min at 72°C; followed by an extension step of 10 min at 72°C. One microliter of this reaction underwent a second round of amplification with a nested 5′ primer (5′-ATACCTCGAGGACTTCACTTCTAATGATGATTATGGG-3′) in exon 10 and the same 3′ primer. The product was visualized on a 1% agarose, 1.5% NuSieve gel. Negative controls included a water control as well as a control which contained patient RNA but no reverse transcriptase.

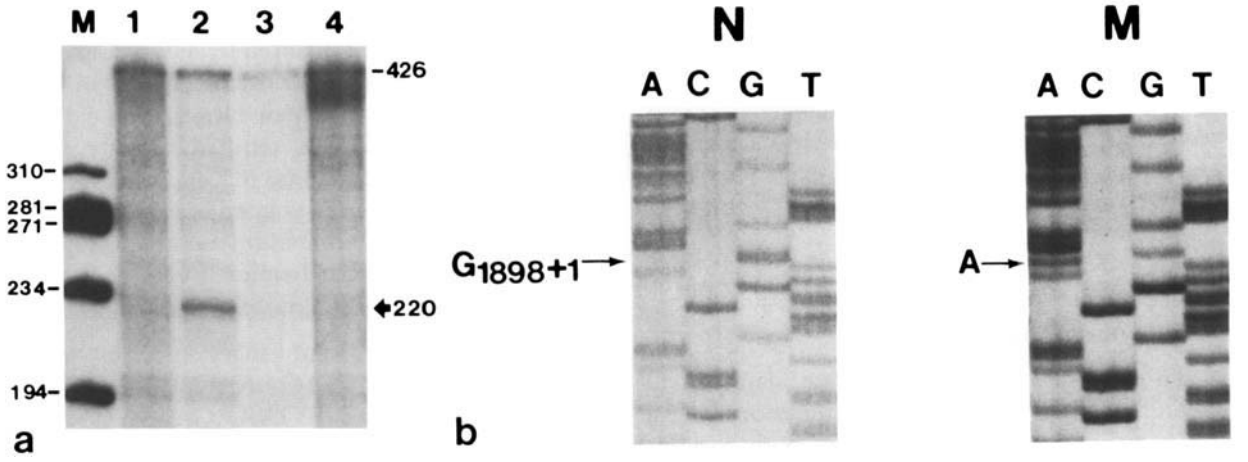

FIGURE 1. Detection of 1898+1G>A mutation by chemical mismatch cleavage and DNA sequence analysis. (a) Heteroduplexes between radiolabeled wild-type probe and amplified DNA from 4 patients were modified with hydroxylamine and cleaved by piperidine. Lanes 1, 3, and 4 show DNA from CF patients with no detectable mutation in the region of exon 12, and lane 2 shows 220 bp cleavage product from patient "A," which indicates the presence of a mutation. DNA markers and their size in base pairs is shown at the left. (b) The partial nucleotide sequence at the exon 12/intron 12 border of an M13 clone from a normal individual (N) on the left, and patient "A" (M) on the right, with the G-to-A transition at base-pair 1898+1 indicated by the arrow.

For amplification of RNA containing exon 12, the first round PCR was performed as above with primers in exons 7 and 13. The nested PCR primers consisted of the 5′ nested primer above, and a 3′ primer complementary to sequences in exon 12 (5′-CACCTGCAGGTTCAAGGAGAGTCTA-ATAAATACAAATCAGC-3′). Reaction conditions were 35 cycles of 1 min at 94°C, 1 min annealing at 60°C, and 2 min extension at 72°C, followed by an extension step of 10 min at 72°C.

Direct Sequencing of PCR Products

Direct sequencing of PCR products was based on the method of Kretz et al. (1989) using a Sequenase Kit. Briefly, PCR products were visualized on a 1% NuSieve GTG gel by long wave ultraviolet light and the bands were excised. The agarose was melted and a 10-μl aliquot was annealed with 50 ng of the 5′ PCR primer, which acted as a sequencing primer. To each DNA: primer tube, diluted dGTP labeling mix, DTT, [^{35}S]dATP, and Sequenase was added according to manufacturer's directions. The reaction was held at 37°C for 4 min, and then 4 μl of the reaction was added to the dideoxy nucleotides. After incubation for 5 min, the reaction was stopped by drying followed by the addition of 6 μl of stop solution, and the samples were electrophoresed on a 6% denaturing gel.

RESULTS

DNA Analysis

CFTR exon 12 from a patient heterozygous for ΔF508 (patient "A") was amplified by PCR and analyzed by chemical mismatch cleavage. A mismatch between the patient's DNA and normal DNA was detected by modification with hydroxylamine and subsequent cleavage with piperidine. The abnormal cleavage product is shown in Figure 1A and indicates the presence of a mutation in the region of exon 12. The 426-bp fragment including exon 12 and the flanking intron DNA was subcloned into M13 and 6 independent clones were sequenced. In three clones, a mutation at the splice donor site immediately following exon 12 was identified; the G of the splice donor site was changed to an A (1898+1G>A) (Fig. 1B). This G is part of the highly conserved GT dinucleotide of splice donor sites. The sequence of the other three clones was normal for exon 12 and the surrounding intron sequences.

To determine if this potential splice mutation was found in any additional patients in our population, DNA from our panel of CF patients was amplified and analyzed by hybridization with an oligonucleotide specific for the mutation. Of 110 non-ΔF CF chromosomes screened, one additional unrelated patient (patient "B") was identified as having the mutation. Sequencing of this patient's

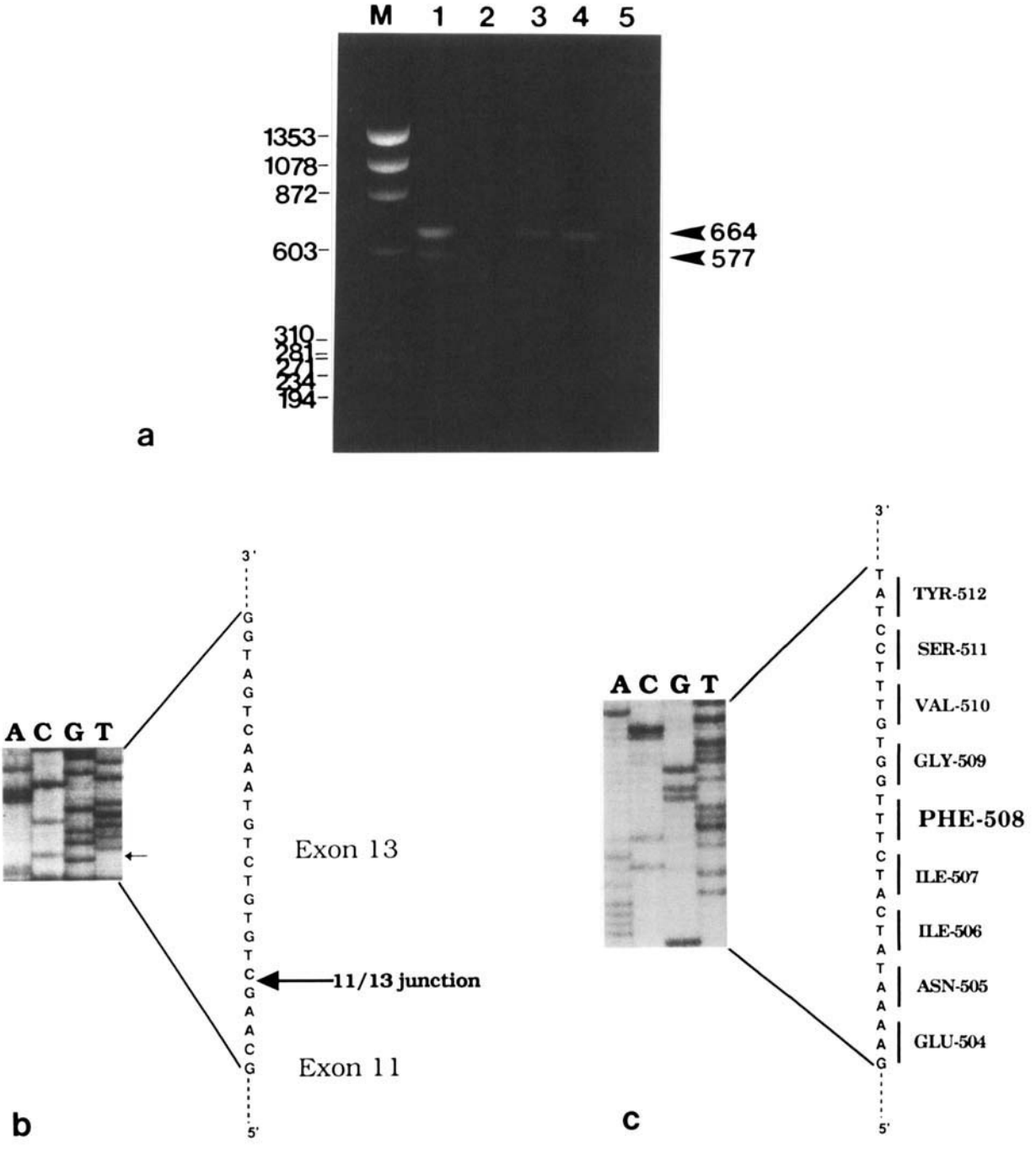

FIGURE 2. Analysis of CFTR mRNA by RT-PCR and direct sequence analysis of the amplification product. (a) RT-PCR analysis. M, DNA marker ϕX174 cut with **HaeIII**. Lane 1. Amplification product from patient "A" nasal epithelium RNA using primers in exons 10 and 13. The abnormal 577 bp band is indicated by the arrow. Lane 2. Negative control in which no reverse transcriptase was added. Lane 3. Amplification product from a control trachea RNA sample. Lane 4. Amplification product from CFTR cDNA. Lane 5. Negative H_2O control. (b) Nucleotide sequence of the 577 bp band demonstrating the junction of exons 11 and 13. (c) Nucleotide sequence of the 577 bp band in the exon 10 region, demonstrating the presence of Phe 508 in the CFTRΔ12 transcript.

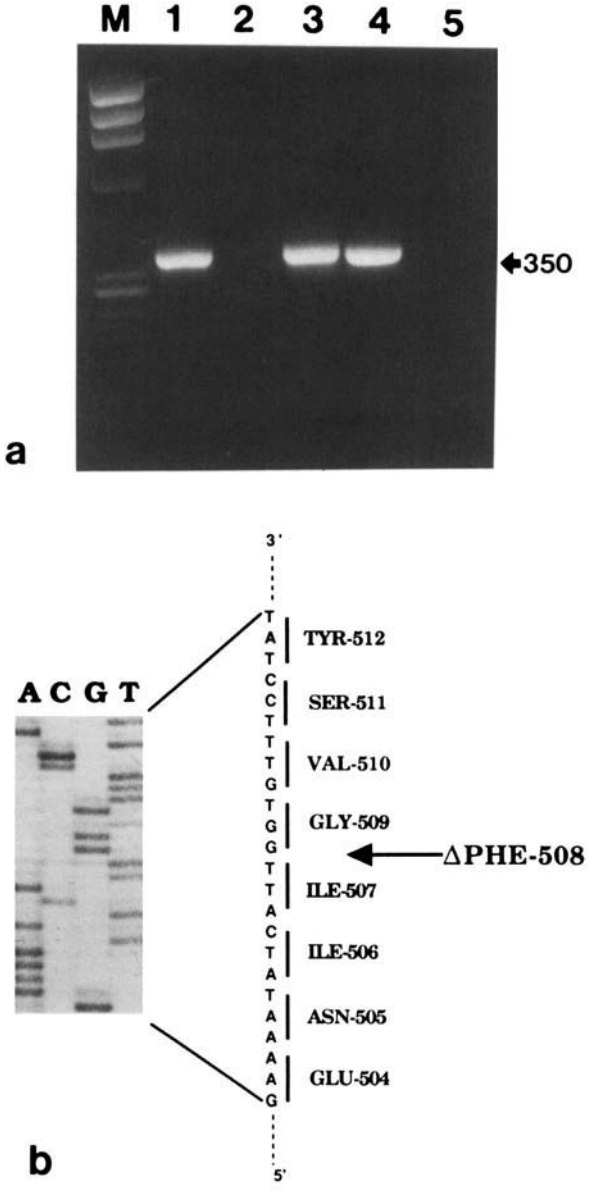

FIGURE 3. Analysis of CFTR mRNA from patient "A" by RT-PCR using a PCR primer complementary to exon 12. (a) M, DNA marker. Lane 1. Amplification product from patient "A" RNA. Lane 2. No reverse transcriptase control. Lane 3. Amplification product from a control trachea RNA sample. Lane 4. Amplification product from CFTR cDNA. Lane 5. Negative H_2O control. (b) Nucleotide sequence of the exon 12 containing amplification product from patient "A" RNA in the region of exon 10. Direct sequencing of the amplification product indicates that no detectable exon 12 containing RNA is transcribed from the 1898+1G>A allele, which is wild type at Phe-508.

DNA confirmed the presence of the 1898 +1G>A change (not shown). This individual was also heterozygous for the ΔF508 allele. Seventy-five normal chromosomes were screened with the ASOs and none were found to have the 1898+1G>A mutation.

RNA Analysis

Since the G of the splice donor site is highly conserved, it seemed likely that a mutation at this site would disrupt normal mRNA processing. To determine the effect of this mutation on the patient's CFTR transcripts, RNA from the nasal epithelium was reverse transcribed and amplified by PCR (RT-PCR) using primers in exons 10 and 13. As shown in Figure 2A, CFTR mRNA from this patient is of two sizes. The relative intensity of the two bands was somewhat variable between experiments, but was approximately equal. Direct sequencing of the smaller RT-PCR product demon-

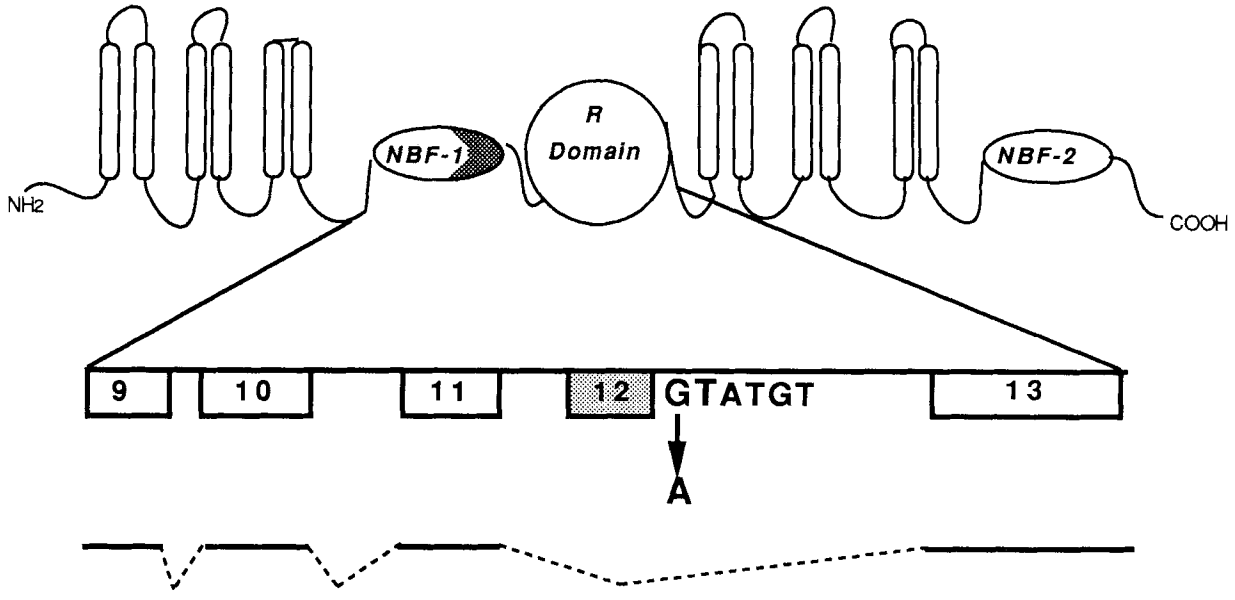

FIGURE 4. **Schematic the CF gene and the predicted protein structure. The location of the 1898+1G>A transition at intron 12 and the mRNA generated as a result of this mutation are shown. The region of CFTR encoded by exon 12 is indicated by the shaded area in the first NBF.**

strated that it represents a transcript that has skipped exon 12, resulting in the joining of exons 11 and 13 (Fig. 2b). This RT-PCR product (CFTRΔ12) was wild type at F508, indicating the aberrantly spliced transcript originated from the chromosome bearing the 1898+1G>A mutation (Fig. 2c). The larger RT-PCR product was also analyzed by direct sequencing (not shown) and predominantly represents the full length message, transcribed from the ΔF508 chromosome. However, this product could not be completely purified by agarose gel electrophoresis and contained a small amount of the 1898+1G>A product, which was wild type at F508 and had exons 11 and 13 spliced together. To confirm that no full length mRNA was transcribed from the chromosome with the 1898+1G>A mutation, PCR was performed on the reverse transcribed RNA using a primer in exon 12. A single band was amplified which originated solely from the ΔF508 chromosome when analyzed by direct sequencing (Fig. 3). A diagram of the location of the 1898+1G>A mutation and its effect on CFTR RNA splicing is shown in Figure 4.

DISCUSSION

Chemical mismatch cleavage and DNA sequencing of CF patient DNA led to the identification of a potential splicing mutation following exon 12 (1898+1G>A). This mutation was found in two of 111 non-ΔF CF chromosomes in our generally North American white CF population. The nucleotide altered in this patient is part of the highly conserved GT dinucleotide found at the splice donor junction sequence. This guanosine is conserved in more than 99.8% of splice sites (Senapathy et al., 1990), indicating a crucial role in mRNA splicing. Mutations in other genes at the critical +1 G nucleotide of the splice donor site have been found to cause disease by at least two different mechanisms. As in the case of the β-globin gene, mutation at this site may result in the activation of a cryptic splice donor site (Treisman et al., 1982). Usually the cryptic splice donor site is located near the splice donor site. A survey of the sequence near the 1898+1G>A mutation revealed the presence of one GT dinucleotide within exon 12 and three within the first 100 bp of the intron that could potentially act as splice donor sites. However, PCR amplification of the transcript indicated that none of these sites was utilized to a detectable degree.

The second, more typical consequence of a 5' splice mutation is a complete skipping of the exon preceding the mutation, as is seen in this case. This mechanism of exon skipping suggests that both the splice acceptor site immediately preceding an exon and the splice donor site immediately following the exon must be recognized before that exon will be spliced into the mature message (Talerico and Berget, 1990).

Reverse transcription and PCR analysis revealed that the effect of the 1898+1G>A mutation is skipping of exon 12, with a precise joining of exons 11 and 13. As assessed by direct sequencing, the full length PCR product originated solely from the ΔF508 chromosome and the CFTRΔ12 product originated from the 1898+1G>A chromosome. The approximately equal quantities of the two messages indicated that the CFTRΔ12 transcript is stable. The stability of this message may be due to the fact that exon 12 consists of 87 bp and its deletion does not result in a shift of reading frame. Therefore, stop mutations, which are associated with unstable transcripts in other CFTR mutants (Hamosh et al., 1991), have not been introduced into the coding region.

Translation of the CFTRΔ12 message would result in the synthesis of a protein which is missing 29 amino acids in the first nucleotide binding fold (Fig. 4). This is a highly conserved region in the family of ATP-binding transport proteins of which CFTR is a member. The region deleted would include the Walker B consensus sequence (Walker et al., 1982), a critical region that is thought to interact with the Mg^{2+} of the MgATP complex (Pai et al., 1990). The resulting protein would be expected to be defective in the binding and hydrolysis of ATP. As hydrolyzable ATP is necessary for CFTR to function as a chloride channel (Anderson et al., 1991), it is unlikely that such a protein, if stable, would be functional. It is interesting to note that we have observed alternative splicing of exon 12 in several tissues from normal individuals (Strong and Collins, in preparation). The finding of this allele which results in the deletion of exon 12 in the mRNA and is associated with disease suggests that the CFTRΔ12 protein, if produced, is not able to carry out all of the normal functions of the full length product.

ACKNOWLEDGMENTS

We wish to thank Lap-Chee Tsui for the gift of normal DNAs. Supported by a Cystic Fibrosis Foundation Research Development Center grant and NIH Grants DK42718 and DK39690 to F.S.C., and DK 27651 to R.C.S. T.V.S. is a Cystic Fibrosis Foundation Postdoctoral Fellow, L.S.S. is a Howard Hughes Predoctoral Fellow, and F.S.C. is a Howard Hughes Investigator.

REFERENCES

Anderson MP, Berger HA, Rich DP, Gregory RJ, Smith AE, Welsh MJ (1991) Nucleoside triphosphates are required to open the CFTR chloride channel. Cell 67:775–784.

Ames GF, Mimura CS, Shyamala V (1990) Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia coli to human: Traffic ATPases. FEMS Microbiol Rev 6:429–446.

Bear CE, Canhui L, Kartner N, Bridges RJ, Jense TJ, Ramjessingh M, Riordan JR (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell 68:809–818.

Boat TF, Welsh MJ, Beaudet AL (1989) Cystic fibrosis. In Scriver CL, Beaudet AL, Sly WS, Valle D (eds): The Metabolic Basis of Inherited Disease, 6th ed. New York: McGraw-Hill, pp 2649–2680.

Cheng SH, Rich DP, Marshall J, Gregory RJ, Welsh MJ, Smith AE (1991) Phosphorylation of the R domain by cAMP-dependent protein kinase regulates the CFTR channel. Cell 66,1027–1036.

Collins FS (1992) Cystic Fibrosis: Molecular biology and therapeutic implications. Science 256:774–779.

Cotton RGH, Rodrigues NR, Campbell RD (1988) Reactivity of cytosine and thymine in single base pair mismatches with hydroxylamine and osmium tetroxide and its application to the study of mutations. Proc Natl Acad Sci USA 85:4397–4401.

Cystic Fibrosis Genetic Analysis Consortium Worldwide survey of the ΔF508 mutation – report from the Cystic Fibrosis Genetic Analysis Consortium. Am J Hum Genet 47:354–359.

Drumm ML, Pope HA, Cliff WH, Rommens JM, Marvin SA, Tsui L-C, Collins FC, et al (1990) Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell 62: 1227–1233.

Grompe M, Muzny DM, Caskey CT (1989) Scanning detection of mutations in human ornithine transcarbamylase by chemical mismatch cleavage. Proc Natl Acad Sci USA 86:5888–5892.

Hamosh A, Trapnell BC, Zeitlin PL, Montrose-Rafizadeh C, Rosenstein BJ, Crystal RG, Cutting GR (1991) Severe deficiency of cystic fibrosis transmembrane conductance regulator messenger RNA carrying nonsense mutations R553X and W1316X in respiratory epithelial cells of patients with cystic fibrosis. J Clin Invest 88:1880–1885.

Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileli U, Pearce SR, Gallagher MP, et al. (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature (London) 364:362–365.

Kerem B-S, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui L-C (1989) Identification of the cystic fibrosis gene: Genetic analysis. Science 245:1073–1080.

Kretz KA, Carson GS, O'Brien JS. (1989) Direct sequencing from low-melt agarose with Sequenase. Nucl Acids Res 17:586–58.

Pai EF, Krengel U, Petsko A, Goody RS, Kabsch W, Wittinghofer A (1990) Refined crystal structure of the triphosphate conformation of H-*ras*p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. EMBO J 9:2351–2359.

Quinton PM (1990) Cystic fibrosis: A disease in electrolyte transport. FASEB J 4:2709–2717.

Rich DP, Anderson MP, Gregory RJ, Cheng SH, Paul S, Jefferson DM, McCann JD, et al. (1990) Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature (London) 347:358–363.

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of the complementary DNA. Science 245:1066–1073.

Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, et al. (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 245: 1059–1065.

Sambrook J, Fritsch EF, Manniatis T (eds) (1989) Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 14.14–14.20.

Senapathy P, Sharpiro MB, Harris NL (1990) Splice junctions, branch point sites and exons: Sequence statistics, identification and applications to genome project. Methods Enzymol. 183:252–278.

Strong TV, Smit LS, Turpin SV, Cole JL, Tom Hon C, Markiewicz D, Petty TL, et al (1991) Cystic fibrosis gene mutation in two sisters with mild disease and normal sweat electrolyte levels. N Engl J Med 325:1630–1634.

Talerico M, Berget SM (1990) Effect of 5′ splice site mutations on splicing of the preceding intron. Mol Cell Biol 10:6299–6305.

Treisman R, Proudfoot NJ, Shander M, Maniatis T (1982) A single base change at a splice in a β^0 thalassemic gene causes abnormal RNA splicing. Cell 29:903–911.

Tsui L-C (1992) Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: A report from the cystic fibrosis genetic analysis consortium. Hum Mut 1:197–203.

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1:945–951.

Welsh MJ (1990) Abnormal regulation of ion channels in cystic fibrosis epithelia. FASEB J 4:2718–2725.

Zielenski J, Rozmahel R, Bozon D, Kerem B-S, Grzelczak Z, Riordan JR, Rommens J, Tsui L-C. (1991) Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics 10:214–228.