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Splicing Factors Induce Cystic Fibrosis Transmembrane Regulator Exon 9 Skipping through a Nonevolutionary Conserved Intronic Element*

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In monosymptomatic forms of cystic fibrosis such as congenital bilateral absence of vas deferens, variations in the TG_mT_n polymorphic repeats at the 3′ end of intron 8 of the cystic fibrosis transmembrane regulator (CFTR) gene are associated with the alternative splicing of exon 9, which results in a nonfunctional CFTR protein. Using a minigene model system, we have previously shown a direct relationship between the TG_mT_n polymorphism and exon 9 splicing. We have now evaluated the role of splicing factors in the regulation of the alternative splicing of this exon. Serine-arginine-rich proteins and the heterogeneous nuclear ribonucleoprotein A1 induced exon skipping in the human gene but not in its mouse counterpart. The effect of these proteins on exon 9 exclusion was strictly dependent on the composition of the TG_m and T_n polymorphic repeats. The comparative and functional analysis of the human and mouse CFTR genes showed that a region of about 150 nucleotides, present only in the human intron 9, mediates the exon 9 splicing inhibition in association with exonic regulatory elements. This region, defined as the CFTR exon 9 intronic splicing silencer, is a target for serine-arginine-rich protein interactions. Thus, the nonevolutionary conserved CFTR exon 9 alternative splicing is modulated by the TG_m and T_n polymorphism at the 3′ splice region, enhancer and silencer exonic elements, and the intronic splicing silencer in the proximal 5′ intronic region. Tissue levels and individual variability of splicing factors would determine the penetrance of the TG_mT_n locus in monosymptomatic forms of cystic fibrosis.

Cystic fibrosis (CF),[1] the most common life-shortening autosomal recessive disorder in Caucasians, is caused by mutations in the CF transmembrane regulator (CFTR) gene and is characterized by pathological features of variable severity at the level of lungs, pancreas, sweat glands, testis, ovaries, and intestine (1). Monosymptomatic forms of the disease such as congenital bilateral absence of vas deference (CBAVD), pancreatitis, nasal polyposis, disseminated bronchiectasies, and broncopulmonary allergic aspergillosis frequently present a peculiar allele at the polymorphic CFTR intron 8-exon 9 junction (2–8). At this locus a variable number of dinucleotide TG repeats (from 9 to 13) followed by a T repeat (T5, T7, or T9) can be found in the normal population, and it has been suggested that the T5 allele is a disease mutation with incomplete penetrance that could be modulated by the simultaneous presence of other mutations and/or polymorphisms (3). The pathologic effect of the T5 allele has been associated to the alternative splicing of the CFTR exon 9, which is extremely variable in humans among different individuals (9). Interestingly, exon 9 skipping is absent in mouse, and it has been reported not to be evolutionary conserved (10). This exon encodes part of the functionally important first nucleotide-binding domain, and its skipping produces a nonfunctional CFTR protein (10, 11). In CBAVD patients and normal subjects, several studies have established a good correlation between the number of TG (3) and particularly T repeats (4, 7, 9) in the polymorphic locus and the amount of CFTR mRNA lacking exon 9. A high number of TG repeats and a low number of T repeats have been shown to favor the exclusion of exon 9 in the mRNA (3). However, the proportion of exon skipping does not correlate in some cases to the polymorphic alleles at the 3′ end of intron 9 (9), and it varies among tissues of the same subject (7). This suggests that other factors operate in conjunction with the polymorphic locus to regulate the amount of exon 9 skipping.

Multiple factors are indeed known to be involved in the regulation of alternative splicing through a complex network of interactions between splicing factors and pre-mRNA elements with both positive and negative effects on the exon recognition. In the last few years, different splicing factors belonging to the serine-arginine-rich (SR) family and heterogeneous nuclear ribonucleoproteins (hnRNPs) have been shown to regulate the alternative splicing of many pre-mRNAs (12–17). In general, SR proteins interacting with specific RNA elements located in exons positively regulate alternative splicing, whereas hnRNPA1 has an antagonistic effect commonly inhibiting splicing (12–18).

The characterization of the splicing factors and *cis*-acting elements involved in the regulation of human CFTR exon 9 alternative splicing is of key importance for the determination of the molecular basis of the skipping and consequently for the

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, CF transmembrane regulator; CBAVD, congenital bilateral absence of vas deference; SR, serine-arginine-rich; hnRNP, heterogeneous nuclear ribonucleoprotein; bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcription; h-int, human intron; m-int, mouse intron; ISS, intronic splicing silencer; ESS, exonic splicing silencer; βTM, β-tropomyosin; TNT, troponin T; mAb, monoclonal antibody; HIV, human immunodeficiency virus.

analysis of the phenotypic variability in patients with monosymptomatic forms of CF. To this end, we have developed an *in vivo* model system consisting of a reporter minigene by means of which the effect of the different CFTR alleles can be experimentally analyzed. With this system we have previously shown that the TG_m and T_n repetitions are directly involved and cooperate in exon 9 skipping and that intron 9 sequences can modulate the alternative splicing. In the present study we have carried out a functional analysis of the role of the regulatory *trans*-acting factors hnRNPA1 and members of the SR family on the alternative splicing of CFTR exon 9.

EXPERIMENTAL PROCEDURES

Hybrid Minigene Constructs—Human genomic DNA was amplified with hcfIVS8dir 5′-ttttcatatggggccgctctaggacttgataatgggcaaatatctta-3′ and hcfIVS9rev 5′-cccctcgaccatatgctcgccatgtgcaagatacag-3′ to generate a fragment that contains exon 9 along with part of the flanking introns (154 bp for intron 8, 183 bp for exon 9, and 209 bp for intron 9). This fragment, which contains two additional *Nde*I sites at the ends, was subcloned in *Sma*-digested pBluescript plasmid. The mouse genomic regions containing exon 9 (154, 183, and 209 bp for intron 8, exon 9, and intron 9, respectively) were amplified from mouse genomic DNA with mCF8idir 5′-ttttcatatgtctagaaaccatgtgctttatagt-3′ and mCF9rev 5′-aaaacatatgataggttatccaatcttaagtgatcagttctaaacacgtgta-3′, which contain additional *Nde*I sites and subcloned in pBluescript plasmid. In both mouse and human constructs, at position +15 of exon 9, an *Eco*RI site was introduced by PCR-mediated site directed mutagenesis (A → C) to facilitate subsequent cloning procedures. By PCR-mediated site directed mutagenesis different T_n and TG_m alleles were introduced at the 3′ end of intron 8 in the human construct with the use of antisense primers 5′-aaagaattccccaaatccctgtt(a)$_n$(ca)$_m$tcaaaaataaaagatgagtt-3′ (19). The construct hmCF was prepared cloning the human *Eco*RI/*Xba*I cassette into the corresponding sites of the mouse construct. hm intron 9 plasmid was created by amplification of the mouse construct with M2CF9dir 5′-ctggatccactggagcaggcaaggtagt-3′ and an external primer in the pBS vector. The resulting fragment was digested with by *Bam*HI/*Kpn*I and subcloned in hCF TG11-T5. hm intron 9 3′ was created by two-step PCR overlap extension with CFPSTdir 5′-ttgtagtgctgcagtatcttactccttccatg-3′ and CFPSTrev 5′-agatactgcagcactacaaactagaa-3′ primers. To generate the expression vectors, we have previously used the α-globin-fibronectin EDA minigene (19). As in this study the FN EDA minigene was used as a control in transfection experiments (see Fig. 2*A*). The CFTR intron 8-exon 9-intron 9 cassette between *Nde*I sites (both for human and mouse) was cloned in a modified version of the α-globin-fibronectin EDB minigene (17). In the original α-globin-fibronectin EDB minigene, the EDB exon along with part of the flanking introns (from *Nde*I in intron −1 to *Xba*I in intron +1) were deleted, and in the unique *Nde*I site created the CFRT inserts were cloned. Human CFTR gene was amplified with the direct primer int/A 5′-ctggatccactggagcaggca-3′ and the reverse primers h-int117 5′-atggtaccatatgtctcctaatgctcatgtaag-3′ or h-int 77 5′-atggtaccatatgccagcactacaaactaga-3′. The resulting fragments, digested with *Bam*HI-*Kpn*I, were subcloned in the corresponding sites of hCF Tg11-T5 to generate hCF Δint1 and hCF Δint2, respectively. Deletions inside CFTR exon9 were prepared by exchanging the *Eco*RI-*Bam*HI cassette with amplified fragments obtained by PCR overlapping method with the following oligonucleotides: Sp1dir 5′-acttctaatggtaccctcttcttcagtaat-3′ with Sp1rev 5′-aagagggtaccattagaagttttctattg-3′ for hCF Δ1 and Sp2dir 5′-tgaaagatatagaaagaggacagttg-3′ with Sp2 rev 5′-ctttctatatctttcaggacaggagt-3′ for hCF Δ2. The constructs hCF Δ2Δint1 and hCF Δ2Δint2 were obtained subcloning the hCF Δ2 *Eco*RI/*Bam*HI exon in the corresponding sites of hCF Δint1 and hCF Δint2, respectively.

Analysis of the Hybrid Minigene Expression—Hep3B cells were transfected with the DOTAP reagent with 3 μg of each reported plasmid and the control empty vector pCG (0.5 μg) (16) or different amounts of splicing factors codifying plasmids. RNA extraction was performed after 48 h, and RT-PCR was done as described (17) with the oligo 2–3 α 5′-caacttcaagctcctaagccactgc-3′ and B2 5′-taggatccggtcaccaggaagttggttaaatca-3′. For quantitation of the PCR reactions, [α-^{32}P]dCTP was included in the PCR reaction mixture, and the products were loaded on 6% native polyacrylamide gel, dried, and exposed to a PhosphorImager. The counts of each splicing band were corrected by the number of C/G present in the PCR product sequence. Because other regulatory elements, like the promoter architecture (20, 21), can significantly affect the splicing pattern, with these hybrid minigene constructs we are looking at relative variations, and the reported exon 9 proportions should not be taken as absolute values occurring *in vivo* in the whole organism.

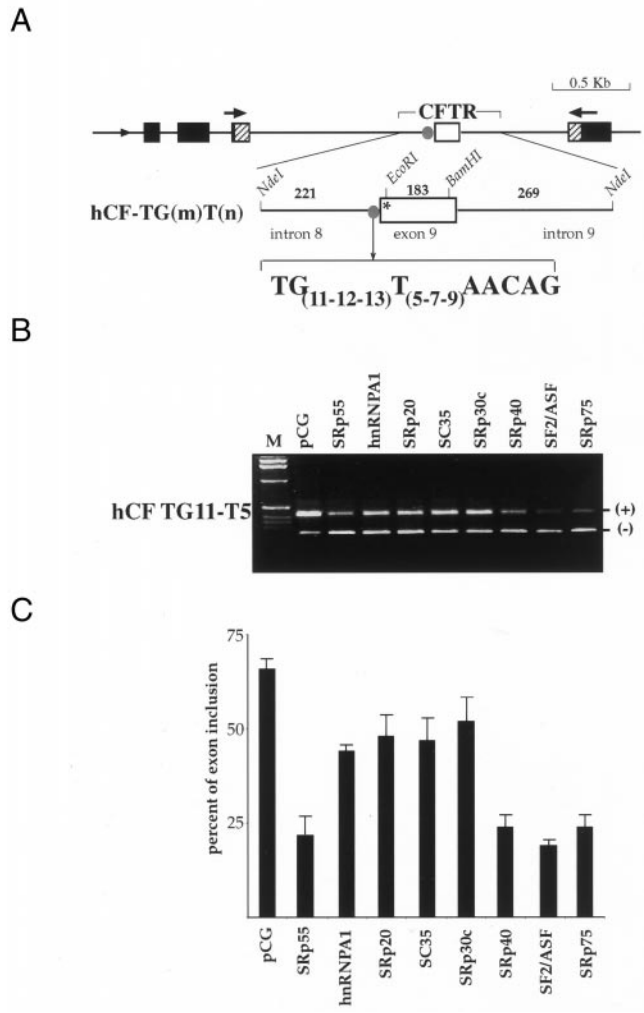

FIG. 1. **SR proteins and hnRNPA1 negatively regulate human CFTR exon 9 splicing.** *A*, schematic representation of the hybrid CFTR exon 9 minigenes. α-Globin, fibronectin EDB, and human CFTR exons are indicated in *black*, *shaded*, and *white boxes*, respectively. The *gray circle* indicates the polymorphic locus. The transcription of the minigenes is driven by a minimal α-globin promoter and SV40 enhancer (*small arrow* at 3′ end). The primers used in the RT-PCR assay are indicated by the *superimposed arrows*. Relevant restriction sites are indicated. The *Eco*RI site, marked with an *asterisk*, was created by site-directed mutagenesis. The length of the relevant CFTR fragments (intron 8, exon 9, and intron 9) is indicated. *B*, expression of the human CFTR exon 9 minigene variant with eleven TG and five T repeats in the presence of different splicing factors. The minigene (3 μg) was transfected in Hep3B cells along with 500 ng of the empty vector pCG (control) or the indicated splicing factor plasmids. RNA splicing variants were detected by RT-PCR and analyzed on a 1.5% agarose gel. Exon 9 positive (+) and negative (−) mRNAs are indicated. *C*, histogram showing the quantification of exon 9 inclusion. The RNA splicing variants, detected by radioactive PCR, were resolved on 6% native polyacrylamide gels and quantitated by using a PhosphorImager. Data are expressed as percentages of exon inclusion and are the means of at least three independent experiments.

UV Cross-linking Assay—To generate the human intron 9 competitor RNAs, human CFTR gene was amplified with the direct primer int/A 5′-ctggatccactggagcaggca-3′ and each of the following reverse oligonucleotides: h-int 5′-atggtaccatatgctcgccatgtgcaagatacag-3′, h-int176 5′-atggtaccatatgaactagagtaaattatcag-3′, h-int117 5′-atggtaccatatgtctcctaatgctcatgtaag-3′, and h-int 77 5′-atggtaccatatgccagcactacaaactaga-3′. Mouse CFTR gene was amplified with int/A and m-int 5′-atggtaccatatgtgatcagttctaaacacgtgta-3′. The PCR products were digested with *Bam*HI/*Kpn*I and subcloned in the same restriction sites of pBS SK plasmid. The UV cross-linking assay was performed by adding [α-^{32}P]UTP-labeled RNA probes (1×10^6 cpm/incubation) for 15 min at

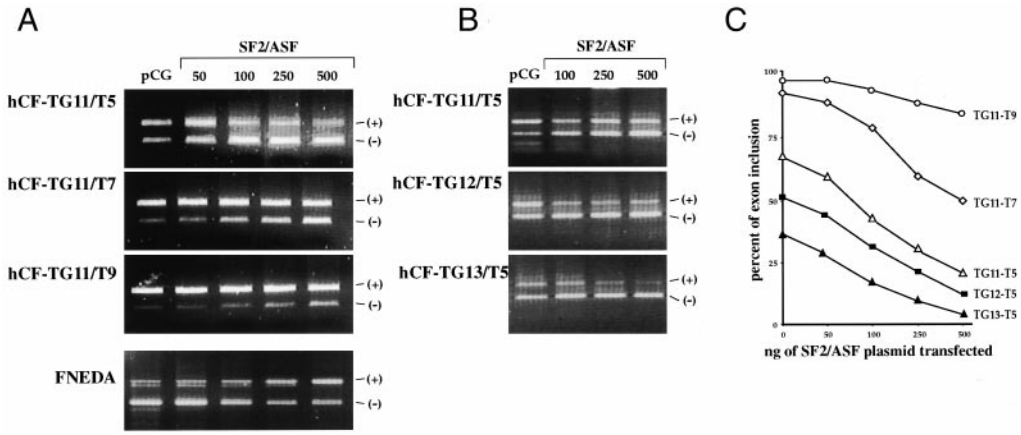

FIG. 2. **Human CFTR exon 9 TG_m and T_n polymorphisms modulate the SF2/ASF-mediated splicing inhibition.** *A*, dose responses of exon 9 splicing by SF2/ASF using minigene variants with different numbers of T repeats. Hep 3B cells were transfected with 3 μg of the indicated minigenes and with 500 ng of the empty vector pCG (control) or with increasing amounts of SF2/ASF plasmid (indicated in ng at the *top* of each *lane*). RNA splicing variants were detected by RT-PCR and analyzed on 1.5% agarose gels. *FNEDA* is a control hybrid minigene that was cotransfected with SF2/ASF and each one of the different CFTR exon 9 minigenes (only one is shown here) and then amplified using specific primers (43). Exon inclusion (+) and exclusion (−) forms are indicated. *B*, dose-responses of exon 9 splicing by SF2/ASF using minigene variants with different numbers of TG repeats. Transfections were performed as in *A*) using the minigenes variants indicated. *C*, SF2/ASF dose-response curves of exon 9 inclusion. The RNA splicing variants detected by radioactive PCR were resolved on 6% native polyacrylamide gels and quantitated using a PhosphorImager. The data belong to the experiments shown in *A* and *B* and are expressed as the percentage of exon 9 inclusion (mean of three independent experiments).

30 °C with 20 μg of HeLa nuclear extracts prepared according to Ref. 22 in 30 μl of final volume. Final binding conditions were 20 mM Hepes, pH 7.9, 72 mM KCl, 1.5 mM $MgCl_2$, 0.78 mM magnesium acetate, 0.52 mM dithiothreitol, 3.8% glycerol, 0.75 mM ATP, 1 mM GTP, and 2 μg of *Escherichia coli* tRNA as a nonspecific competitor. In the competition experiments cold RNA (20-fold molar amount) was also added as a competitor 5 min before addition of the labeled RNAs. Samples were then transferred in the wells of an HLA plate (Nunc, InterMed) and irradiated with UV light on ice (800,000 kJ, approximately 5 min) using a BIO-LINK (Euroclone). Unbound RNA was then digested with 30 μg of RNase A (Sigma) and 6 units of RNase T1 (Sigma) by incubation at 37 °C for 30 min in a water bath. Samples were then analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The sequences of the competitor RNAs TNT, 5′-AAGAGGAAGAAUGGCUUGAGGAAGACGACG-3′ and βTM, 5′-AGGGAAAGACAGGGAGGGAGAGAGAAAGAGAAAGG-3′ are from Refs. 23 and 24.

Electromobility Retardation Assays—To generate the RNA probe, human CFTR exon 9 gene was amplified with the direct primer h77dir 5′-tagagctcggaaggtatttttggagaaattctt-3′ and h-int, and the resulting fragment was subcloned in pBS SK. Electromobility retardation assay was performed by adding [α-^{32}P]UTP-labeled h-int3 RNA probes (1×10^6 cpm/incubation) in a water bath for 15 min at 30 °C with the different protein extracts (both nuclear and SR protein preparations from HeLa cells). In 30 μl of final volume for each experiment, we used 18 μg of total nuclear extract and 1.5 μg of purified SR proteins. SR proteins were prepared from HeLa cells as described previously (25). After 15 min we added to the reaction mixtures the specific mAb hybridoma supernatants followed by the incubation at 30 °C for a further 15 min. The reactions were performed in 1× bind shift binding buffer (50 mM KCl, 10 mM Tris, pH 7.9, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol) in the presence of heparin to a final concentration of 5 mg/ml and electrophoresed on a 6% polyacrylamide gel (acrylamide/bisacrylamide 29:1) at 150 V for 3.5 h in 75 mM Tris-glycine buffer (1 M Tris-glycine buffer is 121.1 g of Tris base, 75 g of glycine, H_2O up to 1 liter) at 4 °C. The gel was then dried on 3MM Whatman filter paper and exposed for 10–20 min with autoradiographic XAR film (Kodak).

RESULTS

The Splicing Factors of the SR Family and hnRNPA1 Induce Exon 9 Skipping—The alternative splicing of many pre-mRNAs is affected by the intracellular concentrations of antagonistic splicing factors of the SR family and hnRNPA1 (12, 14, 16, 17). To evaluate the role of these factors in the regulation of human CFTR exon 9 alternative splicing, we have prepared hybrid minigenes containing this exon as well as part of the flanking introns, including the different polymorphic TG_mT_n alleles (Fig. 1*A*). These variants were inserted in the well characterized α-globin/fibronectin reporter system (17). Different cDNA plasmids coding for SF2/ASF, hnRNPA1, SRp20, SRp30c, SRp40, SRp55, SRp75, and SC35 were transiently expressed in Hep3B cells simultaneously with the transfection of reporter CFTR constructs. The effect of the expression of these regulatory proteins on the splicing of CFTR exon 9 was analyzed by RT-PCR amplification using specific primers. This procedure generates two bands of 239 and 422 bp that correspond to the exclusion or inclusion of exon 9, respectively. In the absence of overexpressed splicing factors, the construct containing TG_{11} and T_5 repeats at the 3′ end of intron 8 produced about 65% of exon 9 inclusion. Overexpression of different splicing factors caused an increase in human CFTR exon 9 skipping (Fig. 1, *B* and *C*). SF2/ASF, SRp40, SRp55, and SRp75 inhibited the most, resulting in only ~25% of mRNA containing the exon 9 (Fig. 1, *B* and *C*). These results provided the first evidence that different SR proteins and hnRNPA1 are important inducers of aberrant human CFTR exon 9 skipping *in vivo*.

The TG_m T_n Polymorphic Variants Modulate the SR Protein-mediated Splicing Inhibition—To analyze the effect of the polymorphic locus at the 3′ end of intron 8 on the negative role of splicing factors, minigene variants with different numbers of TG and T repeats were cotransfected along with the splicing factor SF2/ASF. To evaluate the relative sensitivity to inhibition by SF2/ASF of different alleles, we conducted dose-response studies (Fig. 2). Increasing amounts of SF2/ASF plasmid transfected resulted in a greater amount of CFTR mRNA without exon 9 in all cases. However, the proportion of exon 9 exclusion was strictly dependent on the composition of the polymorphic locus. In fact, the number of TG and T repeats affected independently both basal and splicing factor-induced levels of exon 9 skipping (Fig. 2). For instance, the TG11-T9 construct produced 88% exon 9 inclusion with the addition of 500 ng of SF2/ASF plasmid, which was only 50 and 24% in the case of TG11-T7 and TG11-T5, respectively (Fig. 2, *A* and *C*). On a T5 background, an increasing number of TG repeats further reduced exon 9 inclusion (from 24% for TG11 to 3% for TG13) (Fig. 2, *B* and *C*). Similar dose-response curves were obtained with SRp55 and SRp75, whereas a lower efficiency of

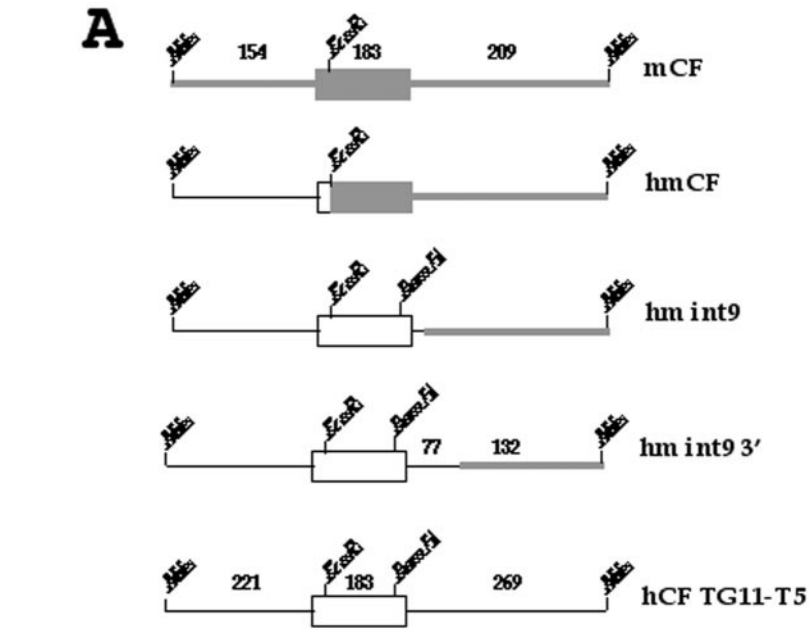

FIG. 3. **Functional comparison between human and mouse sequences regarding splicing inhibition.** *A*, schematic representation of the central portion of minigene variants containing the mouse and human CFTR exon 9 and their hybrids. Mouse CFTR exon and flanking introns are indicated in *gray*. The rest of the symbols are as in Fig. 1. *B*, expression of the human and mouse CFTR exon 9 minigene variants in the presence of the empty vector pCG (−) or increasing amounts of SF2/ASF. Hep3B cells were transfected with 3 μg of minigene plasmids and 250 or 500 ng of splicing factor plasmid.

inhibition was observed for hnRNPA1 (data not shown), suggesting a different mechanism of splicing inhibition by this ribonucleoprotein as recently reported (26). As a control, we cotransfected a hybrid minigene containing the fibronectin EDA exon along with the CFTR and ASF/SF2 constructs. The analysis of the splicing pattern of the EDA exon showed, as previously shown (21), that SF2/ASF produced a dose-dependent increase of exon inclusion, whereas the splicing of the CFTR exon was affected in the opposite way, as described above (Fig. 2*A*). This indicates that the exon skipping of the human CFTR minigene induced by SR proteins is specific to the sequence of exon 9 and/or of its flanking introns and is modulated by the polymorphic locus.

Negative Regulation by Splicing Factors Is Mediated by Sequences Present in Intron 9—To identify the RNA elements in human CFTR mediating the negative regulation induced by the splicing factors, we have compared the human and mouse CFTR genes. As previously reported, the mouse gene is significantly different from the human one within the flanking introns of exon 9. Both TG and T repeats are absent at the intron 8-exon 9 junction, and in addition there are substantial sequence differences in the intron 9 (19). The mouse CFTR exon 9 and its flanking sequences were cloned in the same minigene construct used for the human exon and transfected along with increasing amounts of SF2/ASF splicing factor. Contrary to the human counterpart, the mouse CFTR exon 9 was not significantly skipped in the presence of SF2/ASF overexpression (Fig. 3, *A* and *B*, *mCF*). This result is consistent with the observed lack of alternative splicing of this exon (10). We then prepared hybrid minigene constructs with parts of the human homologue inserted in the mouse context. These mouse-human hybrids were transiently transfected along with increasing amounts of SF2/ASF. The human intron 8 with the polymorphic repeats did not confer an SR protein-mediated inhibitory effect to the mouse exon, indicating that the human polymorphic tract is not by itself the target of this splicing factor (Fig. 3, *A* and *B*, *hmCF*). When intron 9 of the human construct was substituted with the corresponding intron from mouse, overexpression of SF2/ASF did not induce exon 9 skipping (Fig. 3, *hm int9 3′*). Similar results were obtained with SRp55 and SRp75 (data not shown). These data indicate that the inhibitory splicing effect of SR proteins is mediated by sequences present in the human but not in the mouse intron 9.

Functional Analysis of Intronic and Exonic Splicing Regulatory Elements Mediating the SF2/ASF Splicing Inhibition—To evaluate the functional significance of the human intron 9 in mediating the splicing inhibition the intronic sequences were progressively deleted and the corresponding hybrid minigenes

transiently transfected in Hep3B cells. The partial or complete deletion (up to 77 bp from the 5′ splice site) of the intronic sequences leads to the complete disappearance of the CFTR exon 9 minus form, indicating that this regions behave like an intronic splicing silencer (ISS). When increased concentration of SF2/ASF plasmid were cotransfected, the CFTR exon 9 minus form was induced at a significant lower level than the amount present in the minigene, which contains the entire intronic element (Fig. 4, *minus lanes* for Δint1 and Δint2). The splicing inhibition was related to the length of the deletion of the intron, suggesting the presence of multiple regulatory elements with inhibitory properties. These data are consistent with the role of the ISS in mediating the inhibitory activity, although the data also indicate that this is not the only element involved. Exonic regulatory elements have been found in different alternative spliced genes; hence we evaluated the presence of such elements in the CFTR exon 9 and their putative role in splicing inhibition. Deletion analysis was carried out on exon 9 selected chosen taking into account the RNA secondary structure of this region (19), which in some cases have been found to be of critical importance (17). Cotransfection experiments in Fig. 4 identified two key exonic regulatory sequences behaving like an exonic splicing enhancer (hCFΔ1) and an exonic splicing silencer (hCFΔ2), respectively. Both elements modulate the response to SF2/ASF splicing inhibition. In fact the splicing inhibition mediated by SF2/ASF was completely prevented when both the exon (ESS) and the intron (ISS) silencers were deleted. Similar results were obtained with SRp55 and SRp 75 (data not shown). These data suggest that the two silencer elements are necessary for the splicing inhibition mediated by SR proteins.

The Intronic Splicing Silencer Element Binds to SR Proteins—The human and mouse intron 9 sequences have a strikingly different behavior regarding the SR proteins inhibitory splicing effect. We have tested the ability of the these two intronic sequences to interact with nuclear proteins using an UV cross-linking assay with specific constructs (Fig. 5*A*). Fig. 5*B* shows that human and mouse intron 9 have a different pattern of protein binding in the UV cross-linking assay (Fig. 5*A*). In particular three bands in the range of 35–44 kDa and to a lesser degree a band of ~75 kDa do not interact with the mouse homologue (Fig. 5*B*, *bands a–d*). Competition experiments using truncated human intron 9 RNA sequences show that the binding of one of the proteins in the 35–44 kDa range (*bands d*) requires a ~150-bp region located between 117 and 264 bases downstream of the 5′ splice site that we named CFTR exon 9 ISS (Fig. 5, *B* and *C*). The band of ~75 kDa (*band a*) was only partially competed by h-int 176 and h-int. The molecular masses of these proteins are consistent with those of some of the SR proteins that have an effect in the functional assay and in particular the *d band*, which molecular weight is similar to SF2/ASF and/or SC35 when cross-linked to RNA (see above). In an attempt to characterize them, we have used competitor RNA sequences derived from β-tropomyosin (βTM) and TNT RNAs, which are known to bind specifically to SF2/ASF, SC35 and SRp75 (23, 24). The UV cross-linked *d band* in the 35–44-kDa range was specifically and completely competed by the βTM and TNT SR binding sequences (Fig. 5*C*, *lanes βTM* and *TNT*).

To address more directly the interaction of cellular factors with the ISS element, we have performed gel shift assay with both nuclear extracts and purified SR proteins (Fig. 5*D*). The ISS RNA transcript formed stable complexes with both nuclear extract and SR proteins that were disrupted by the coincubation with the anti-SR antibody mAb104, indicating that SR proteins contributed to the shift of the ISS element. The addition of a nonspecific antibody had no effect on the migration of the complex. This effect mediated by mAb104 has been previously observed in the NCAM E17 exon (27). These data, together with the cotransfection experiments, indicate that the interaction of SR proteins with the ISS element contribute to the exon 9 splicing inhibition.

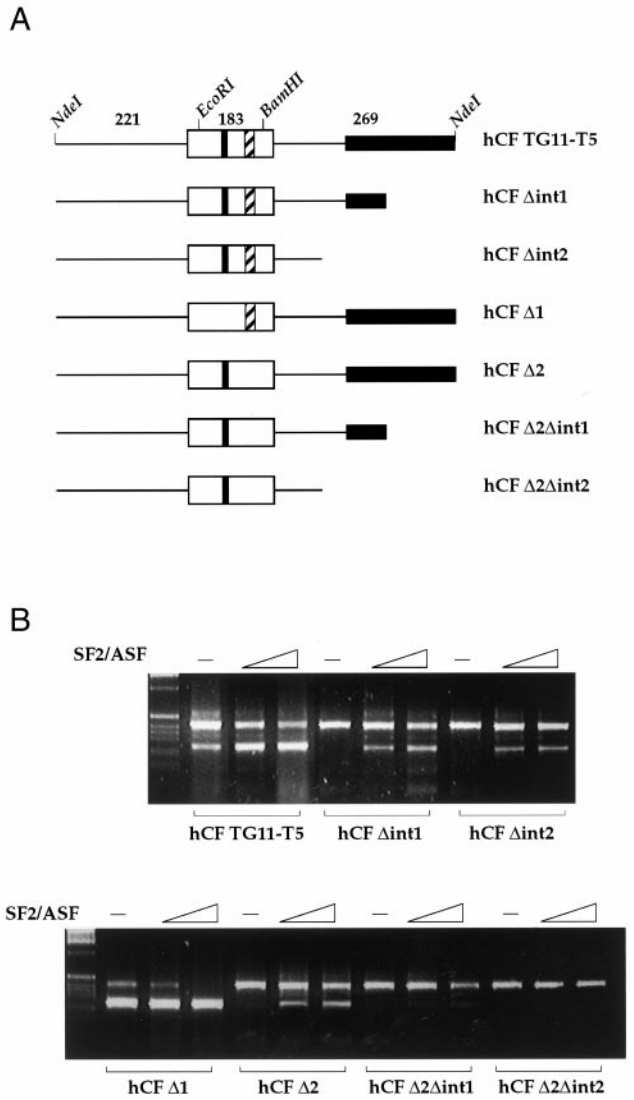

FIG. 4. **Functional identification of intronic and exonic splicing regulatory elements in the CFTR exon 9 minigene constructs mediating the SF2/ASF splicing inhibition.** *A*, schematic representation of the central portion of human CFTR exon 9 minigene variants. Splicing enhancer (GAUGAC) and silencer (UUAAUUUCAAGA) sequences in CFTR exon 9 are shown as *gray* and *outlined boxes*, respectively, whereas the *black box* in intron 9 corresponds to the intronic splicing silencer. *B*, expression of the human CFTR exon 9 minigene variants in Hep3B cells in the presence of the empty vector pCG (−) or increasing amounts of SF2/ASF splicing factor vector (250 and 500 ng).

DISCUSSION

Our results demonstrate that the alternative splicing of human CFTR exon 9 is negatively regulated by the intracellular concentration of different splicing factors and modulated by a number of *cis*-acting elements, the number of polymorphic TG and T repeats, the exonic splicing regulatory regions (exonic splicing enhancer (ESE) and ESS), and the ISS. The splicing factors affecting CFTR exon 9 alternative splicing belong to two different groups of RNA binding proteins, the SR proteins and hnRNPs. SR proteins in general are activators of splicing (12–

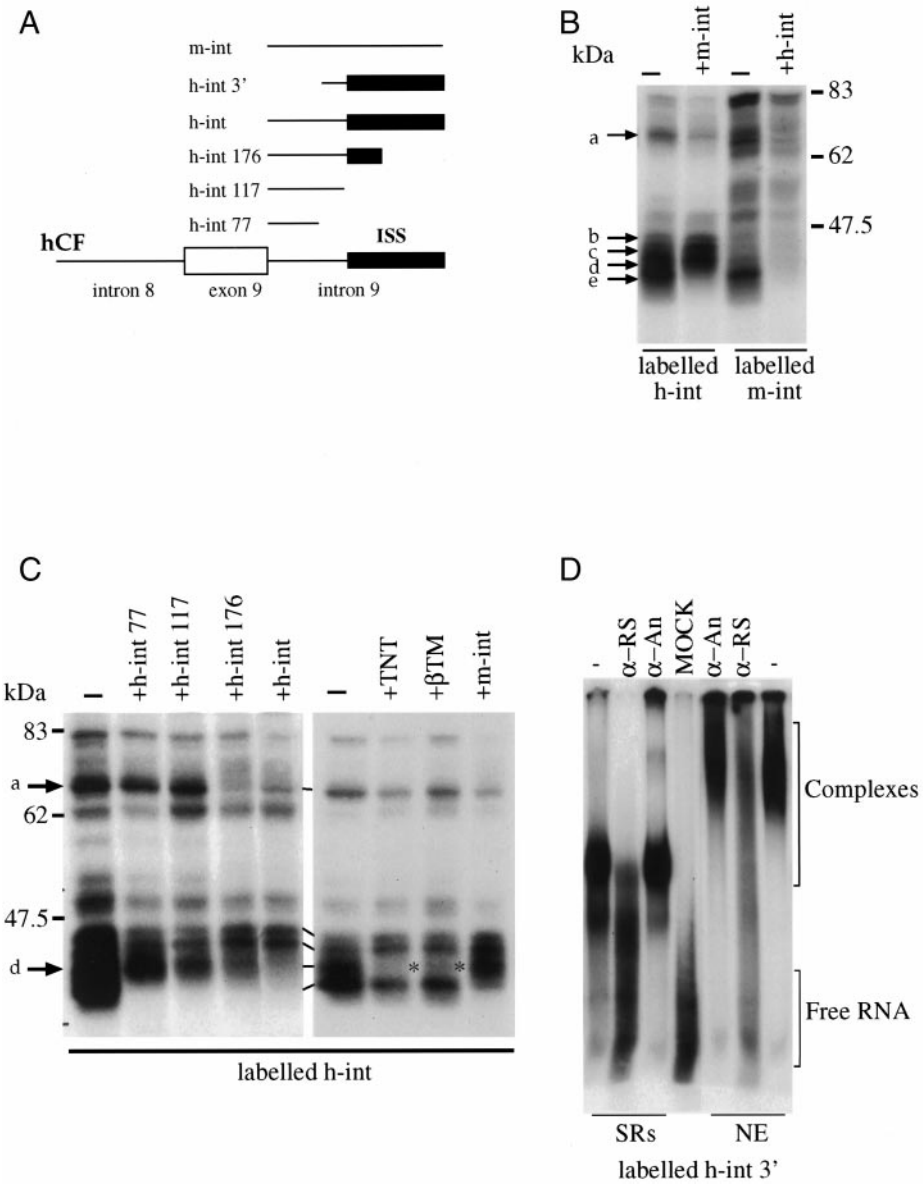

FIG. 5. **The CFTR exon 9 ISS element of human intron 9 binds SR proteins.** *A*, schematic representation of human CFTR exon 9 showing the location of the RNAs used in the UV cross-linking experiments and in gel shift assay. The ISS element in the intron 9 of the human CFTR gene is shown as a *black box*. *B*, UV cross-linking assay of HeLa nuclear extracts with human and mouse intron RNAs (h-int and m-int, respectively) challenged with the indicated competitors. Four UV cross-linked bands, one at ~75 kDa (*a*) and three in the 35–44-kDa range (*b–d*) bind to the human (h-int) and not to the mouse (m-int) intron 9. The bottom band (*e*) binds to both the mouse and human introns. Specific binding to the human intron is shown for the *a* and *c* proteins as they can be competed by the human cold RNA only (*B*, *lane +h-int*) but not by the cold mouse RNA (*lane +m-int*). *C*, competitor RNAs h-int 77, h-int 117, and h-int 176 correspond to truncated human intron 9 RNA sequences extending 77, 117, and 176 bases from the 5′ splice site on exon 9 (whereas the total length of h-int is 269 bases). The *d band* in the 35–44-kDa range and the *a band* of 75 kDa are entirely competed by the complete intron h-int and by h-int 176, partially competed by h-int 117 and not competed at all by h-int 77 or m-int. βTM and TNT are RNAs containing well characterized strong SR binding sites (23) (24). These RNAs specifically compete the *d band* in the 35–44-kDa range whose absence is indicated by an *asterisk*. *D*, gel shift assay with nuclear extracts (*NE*) and purified SR proteins from HeLa cells using h-int3′-labeled RNA. The position of free and bound complexes are shown. *α-SR* is a monoclonal antibody that recognize phosphorylated SR proteins (mAb104), whereas *α-An* is an aspecific monoclonal antibody.

17), although they can act as repressors depending on their position of binding to the pre-mRNA (18, 28). For example adenoviral IIIa splicing is repressed by an SR protein binding to an intronic repressor element located immediately upstream of the 3′ splice site (18). Nonproductive interactions of SF2/ASF and the small nuclear ribonucleoproteins U1, U2, and U11 at the negative regulator of splicing element are responsible for splicing inhibition in Rous sarcoma virus (28–30). We show here that a protein complex containing SR proteins binds to the ISS element in the CFTR intron 9 (Fig. 5) and repress splicing. SR proteins-ISS complex could interfere with the recruitment of essential splicing factors at the adjacent 5′ splice site of intron 9, resulting in the observed splicing inhibition. Alternatively, SR proteins binding to the ISS element could modulate pre-mRNA conformation by interacting with other regulatory elements bound to the region of the TG and T repeats at the 3′ end on intron 8 or in the exon. Regarding the latter further studies are needed to characterize the enhancer and to determine whether the CFTR exon 9 ESS acts as a target for SR-RNA interactions or whether, as is the case of the fibronectin EDA exon, the ESS only modulates RNA conformation enhancing SR binding on distant target sequences (17). The other splicing factor analyzed, hnRNPA1, has a general inhibitory effect on splicing (14, 16), and this is also the case for the

human CFTR exon 9 (Fig. 1). It has been proposed that the mechanism of action of this splicing factor involves changes in mRNA secondary structure (26). In our case, we have made the unexpected observation that both splicing factors, which frequently have antagonistic effects (12–17), inhibit CFTR exon 9 splicing.

In patients with atypical CF, some of the phenotypic variability can be due to an aberrant regulation of CFTR exon 9 alternative splicing mediated by tissue-specific and/or developmentally controlled changes in the concentration of splicing factors. In monosymptomatic forms of CF, like CBAVD and disseminated bronchiectasies, the partial penetrance of the well studied T5 allele at the polymorphic locus can be modulated not only by the TG repeats upstream, as previously suggested (3), but also by a variability in the individual tissue concentration of splicing factors (12, 31–36). Relatively higher amounts of both SR proteins and hnRNPA1 are expected to negatively affect the recognition of CFTR exon 9 and result in its skipping with the subsequent development of a tissue-specific CFTR defect. This would be particularly apparent with low T and high TG repeat numbers at the polymorphic intron 8-exon 9 junction. In fact overexpression of only one splicing factor could produce up to 97% of exon 9 skipping (Fig. 2). Splicing factors could induce exon 9 skipping during organ development in tissues where CFTR is functionally important and not necessarily during the adult age, as in the case of CBAVD.

Aberrant regulation of CFTR exon 9 alternative splicing mediated by splicing factors could represent a new mechanism causing disease in humans. Recently, aberrant splicing in the absence of any alteration in the DNA sequence has been found in the EAAT2 glutamate transporter mRNA. The presence of this particular defect only in neuropathologically affected areas of the brain in amiothrophic lateral sclerosis has led to the suggestion that an RNA-binding protein with tissue-specific expression could be responsible for the disease (37, 38).

The inhibitory effect of the splicing factors in the human CFTR exon 9 mediated by the nonevolutionary conserved ISS intronic element could be the result of the activity of some ancient transposable elements in the human lineage (39). In fact, in mammalian genomes are abundant traces of recombination events via retrotransposons or retroviruses that resulted in substantial changes in specific regions of the genome (40). In the case of CFTR, scars of a retrotransposon interaction can be seen in the larger size of the introns flanking human exon 9, the peculiar repetitive TG and T sequences, the ISS element, and the amplification of exon 9 sequences found in different chromosomes throughout the genome (39). It is noteworthy that SF2/ASF has been implicated in the negative regulation of retroviral splicing in Rous sarcoma virus and HIV-1 intronic sequences (30, 41, 42). A fascinating hypothesis would be to consider the human CFTR exon 9 ISS as a reminiscence of functionally similar retroviral sequences accidentally left by retrotransposition and amplification events in the human genome. The results presented in this paper indicate for the first time that SR proteins can interact with an intronic element and modulate the penetrance of a disease-causing mutation.

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Splicing Factors Induce Cystic Fibrosis Transmembrane Regulator Exon 9 Skipping through a Nonevolutionary Conserved Intronic Element

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