Missense, Nonsense, and Neutral Mutations Define Juxtaposed Regulatory Elements of Splicing in Cystic Fibrosis Transmembrane Regulator Exon 9*

Franco Pagani, Emanuele Buratti, Cristiana Stuani, and Francisco E. Baralle‡

From the International Centre for Genetic Engineering and Biotechnology, Padriciano 99, Trieste 34012, Italy

Received for publication, December 17, 2002, and in revised form, April 17, 2003
Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.M212813200

Exonic sequence variations may induce exon inclusion or exclusion from the mature mRNA by disrupting exonic regulatory elements and/or by affecting a nuclear reading frame scanning mechanism. We have carried out a systematic study of the effect on cystic fibrosis transmembrane regulator exon 9 splicing of natural and site-directed sequence mutations. We have observed that changes in the splicing pattern were not related to the creation of premature termination codons, a fact that indicates the lack of a significant nuclear check of the reading frame in this system. In addition, the splice pattern could not be predicted by available Ser/Arg protein matrices score analysis. An extensive site-directed mutagenesis of the 3′ portion of the exon has identified two juxtaposed splicing enhancer and silencer elements. The study of double mutants at these regulatory elements showed a complex regulatory activity. For example, one natural mutation (146C) enhances exon inclusion and overrides all of the downstream silencing mutations except for a C to G transversion (155G). This unusual effect is explained by the creation of a specific binding site for the inhibitory splicing factor hnRNPH. In fact, on the double mutant 146C-155G, the silencing effect is dominant. These results indicate a strict dependence between the two juxtaposed enhancer and silencer sequences and show that many point mutations in these elements cause changes in splicing efficiency by different mechanisms.

The correct definition of exonic and intronic sequences includes not only the recognition of discrete elements at the 3′ and 5′ splice sites, the polypyrimidine tract, and the branch site, but also the contribution of less well defined exonic cis-acting elements, which may influence the use of the flanking splice sites. Exonic sequences have been shown to modulate the splicing efficiency, and single base changes in these sequences may cause pathological splicing events by inducing exon skipping or exon inclusion (1–4). Several mechanisms have been proposed to explain the splicing regulation mediated by these exonic sequences. In some cases, the creation of nonsense mutations has been shown to induce exon skipping, leading to the proposal of a putative nuclear reading frame scanning mechanism for nonsense codons and consequent elimination of the exons (1, 5–9). In addition, the well established nonsense-mediated decay of mRNAs containing nonsense codons (10, 11) can also be involved in selective degradation of nonsense alternatively spliced mRNAs. However, not all mRNAs are subjected to nonsense-mediated decay (12, 13).

Skipping of constitutive exons may also occur for missense and silent mutations. In this case, disruption of exonic splicing enhancer or the creation of exonic splicing silencer has been considered an alternative mechanism to explain the exon skipping phenotype (2, 10, 14, 15). Some exonic splicing enhancers in pre-mRNAs interact with serine/arginine-rich proteins (SR proteins) (16). SR proteins are essential splicing factors required for both constitutive and alternative splicing, and changes in their relative concentrations with respect to antagonistic splicing factors have been found to affect splice site selection (17). Functional systematic evolution of ligands by exponential enrichment (SELEX) strategies have identified highly degenerate consensus sequences binding to SR proteins. These sites are more represented in exons than in introns (18, 19). In the BRCA1 and SMN genes, changes in SR protein score motifs derived from these SELEX experiments at exonic splicing enhancer sites have been shown to correlate with the efficiency of splicing (15, 20). This has led to the suggestion that SR protein score motif analysis might represent a useful tool to identify general controlling elements of splicing efficiency. On the other hand, exonic splicing silencer elements have always been classically separated from enhancer sequences and may interact with negative regulators, which often belong to the heterogeneous nuclear ribonucleoprotein (hnRNP) family. In particular, binding of hnRNPH at G-rich sequences has been recently found to exert a strong inhibitory splicing effect in the rat β-tropomyosin gene, in the Rous sarcoma virus, and in human immunodeficiency virus-1 (21–24).

The CFTR exon 9 alternative splicing represents an interesting model to evaluate the contribution of exonic sequences in normal and pathologic pre-mRNA processing. Exon skipping produces a nonfunctional CFTR protein, and alternative splicing of this exon has been associated with monosymptomatic forms of cystic fibrosis (CF). The cis-acting elements so far identified include the polymorphic region at the 3′-end of intron 8 and the intronic splicing silencer in intron 9. The polymorphic locus contains a variable number of dinucleotide TG (from 9 to 13) followed by a T repeat (T5, T7, or T9), and a high number of TG repeats and a low number of T tract induce exon skipping.

* This work was supported by grants from the Telethon Onlus Foundation and the “Associazione Italiana Ricerca Cancro” (to F. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 39-040-3757337; Fax: 39-040-3757361; E-mail: baralle@icgeb.org.

The abbreviations used are: SR protein, serine/arginine-rich protein; CERES, composite exonic regulatory element of splicing; CF, cystic fibrosis; hCF, human CF; CFTR, cystic fibrosis transmembrane regulator; SF2/ASF, splicing factor 2; hnRNPH, heterogeneous ribonucleoprotein; hnRNP, heterogeneous ribonucleoprotein H; SELEX, systematic evolution of ligands by exponential enrichment; RT, reverse transcription; WT, wild type.

This paper is available on line at http://www.jbc.org

26580
through the entire exon. These substitutions correspond to previously reported seven missense and one nonsense mutations found in classical CP and/or associated with phenotypes of different severity with a tissue specific involvement. Normal or mutated exon 9 sequences were thus inserted in the previously reported hybrid minigene, which has been widely utilized to study alternative splicing regulation of different genes (Fig. 1A) (25, 28–30), and in the case of the CFTR exon 9, it has been shown to mimic the endogenous splicing pattern (26, 27). Since CFTR exon 9 splicing efficiency is modulated by the composition of the polymorphic locus at the 3′-end of intron 8, we first used minigenes with TG11 and T5 repeats. The minigenes were transiently transfected into Hep3B cells, and the splicing pattern was analyzed by RT-PCR amplification. As shown in Fig.

**EXPERIMENTAL PROCEDURES**

**Hybrid Minigene Expression Analysis**—The natural and artificial point mutations were introduced in the previously described hCF-TG(T) minigenes (26) between the EcoRI and BamHI sites, which were substituted with the appropriate EcoRI-BamHI cassettes created by PCR-mediated site directed mutagenesis. In hCF, due to the context of the minigene, the natural reading frame of exon 9 (183 bp) was shifted by 1 nucleotide, introducing several stop codons. In the F1 construct, the deletion of a nucleotide in position 10 (T16) and the insertion of a G at position 184 (G144 +1) produce an open reading frame with both the −1 and +1 exons of the minigene. In the F2 construct, the exon 9 was also placed in an open reading frame by the elimination of a stop codon at the 5′-end of the exon (A20G, TGA/G11032) for either the endogenous or artificial promoters. The oligonucleotides used for PCR-mediated mutagenesis are shown in Table 1. The PCR products were verified by sequence analysis. Minigene expression analysis was performed on Hep3B cells that were transiently transfected with 3 μg of each minigene plasmid with DOPAT (N,N,N′-trikoethyleneoxypropyl)-N,N,N′,trimethylammonium methyl-sulfate (Roche Applied Science) and, when indicated, with 500 ng of SP2/ASF plasmid. Forty-eight hours post-transfection, RT-PCR was carried out on total RNA as previously described (26) with the primers 2–8 and B2. Cycloheximide (Gibco) or actinomycin D (Roche Applied Science) were added 48 h post-transfection for cycloheximide treatment or, 8, and 24 h post-transfection for actinomycin D. PCRs were optimized to remain in the exponential range of amplification, and products were routinely fractionated on 1.5% agarose gel. For quantitation of the PCRs, the RNA Transcription, UV-cross-linking Assay, and Cross-linking of RNA to Adipic Dehydrazide-agarose Beads—To generate the WT and mutant RNAs, the different CFTR exon 9 hybrid minigenes were first amplified with the direct primer 5′-atggtatataaatcagttcgtgacaggtt-3′, which contains a T7 polymerase sequence and the reverse primer 5′-ctgctggcttgactacg-3′ followed by in vitro T7 RNA polymerase transcription. The amplified region corresponds to the last 64 bp of CFTR exon 9. Transcription of labeled RNAs, nuclear extract preparation, and UV-cross-linking assay were performed as previously described (26, 27). Cold substrate RNAs for bead immobilization were synthesized by in vitro transcription using T7 RNA polymerase, and cross-linking of RNA to adipic dehydrazide-agarose beads was done essentially as previously described with the addition of heparin to a final concentration of 5 μg/μl (26, 27). Proteins were separated on a 10% SDS-PAGE gel and visualized by Coomassie Brilliant Blue staining. The gel was destained onto a nitrocellulose membrane and probed with a rabbit polyclonal anti-hnRNPH antiserum. Immunoblotts were detected using the ECL chemiluminescence kit (Pierce).

**RESULTS**

**CFTR Exon 9 Natural Substitutions Can Affect the Splicing Efficiency**—To evaluate the contribution of exonic elements in the regulation of CFTR exon 9 alternative splicing, we studied, in the first instance, eight natural point mutations distributed

(25–27). TDP43 binding to the UG polymorphic repeat reduces the proper recognition of the nearby 3′ splice site and in association with the intronic splicing silencer element in intron 9 mediates exon skipping (27).

In this paper, we have analyzed the role of CFTR exon 9 sequences, taking advantage of several natural missense and nonsense mutations and extending them by extensive site-directed mutagenesis. Our results indicate that CFTR exon 9 splicing is extremely sensitive to small variations in its exonic sequence, suggesting that the entire sequence of the exon is important for exon recognition and processing, which occur independently from the maintenance of an open reading frame within the mRNA. We also identify at the 3′ portion of the exon a composite regulatory element with juxtaposed enhancer and silencer properties.
1B, some of the natural substitutions, when compared with wild type hCF, significantly modify the splicing pattern. Four of the natural substitutions, C31T (Q414X), G61A (G424S), T122G (I444S), and C155A (A455E), significantly decreased exon 9 inclusion to 48, 30, 40, and 16%, respectively, whereas only a modest decrease was evident for N418S. The G118T (D443Y) and G157T (V456F) mutations did not significantly affect the splicing pattern, whereas the A146C (Q452P) caused an almost complete inclusion on the exon (96%). It is interesting to note that three nearby mutations, A146C, C155A, and G157T, at the 3’ portion of the exon have a completely different effect on splicing.

To evaluate the effect of exonic substitutions in relation to the strength of the 3’ splice site, we took advantage of the presence of the polymorphic TG and T repeats, which have been shown to modulate the efficiency of the exon recognition. The splicing efficiency of the missense and nonsense variations was analyzed not only in the TG11T5 construct but also in the TG11T9 and TG13T5 minigenes, two variants that cause a respective increase and decrease in the splicing efficiency. Each mutation showed a percentage of exon 9 inclusion to be higher in the presence of the TG11T9, intermediate in the TG11T5, and lower in the TG13T5 context (Fig. 1C). This indicates that both the splicing controlling elements affected by the missense and nonsense mutations along with the 3’ splice site definition independently contribute to the overall efficiency of CFTR exon 9 splicing.

**Reading Frame Contribution to CFTR Exon 9 Alternative Splicing**—Nonsense mutations have been found in some cases to induce abnormal skipping of the exon. We have evaluated the possibility that some of the changes in the splicing pattern induced by the natural substitutions in exon 9, particularly the Q414X, might be related to nonsense mediated altered splicing. The two mechanisms suggested to be involved in the skipping of exons carrying stop codons are a putative nuclear open reading frame scanning process (31) and the contribution of exonic splicing regulatory elements (15). We evaluated the role of these two mechanisms in the CFTR exon 9 using our minigene system that provides a particular advantage, since alternative splicing produces one mRNA always in an open reading frame (the CFTR exon 9− form) and one mRNA that can contain stop codons according to the reading frame and to the mutation introduced (CFTR exon 9+) form. In the construct containing the normal exon 9 (hCF), the processed CFTR exon 9− mRNA is in frame; however, the CFTR exon 9+ mRNA contains numerous stop codons (Fig. 2A). This is due to the nonnatural context of the minigene, where the presence of globin-fibronectin exons results in a one-nucleotide shift of the reading frame. Transfection of this minigene showed about 65% of exon inclusion. To restore an open reading frame in the minigene that results in the elimination of the multiple stop codons, we prepared the F1 and F2 constructs (Fig. 2A). The open reading frame in both of the mRNAs produced by these minigenes was restored by a base deletion at the 5’-end (F1 contains the T16 deletion, and F2 contains an A23 deletion along with an A20G substitution) and a G insertion at the 3’-end (G164+) (for details, see “Experimental Procedures”). Cotransfection of these two constructs produces about 80% of exon inclusion (Fig. 2D, lanes F1 and F2, respectively). To exclude the possibility that this increase in exon recognition may be due to differential mRNA stability of one of the two alternatively spliced mRNAs (with or without exon 9), the cells were treated with cycloheximide or actinomycin D. Cycloheximide, impairing the efficiency of translation, has been shown to inhibit nonsense-mediated decay (32–34). Analysis of the hCF, F1, and F2 constructs did not show any difference on the relative abundance of the two mRNA variants when treated with the translation inhibitor or actinomycin D (Fig. 2, B and C). This indicates that differential stability of the mRNAs in

![FIG. 2. Evaluation of CFTR exon 9 nonsense-associated altered splicing in hybrid minigene experiments.](http://www.jbc.org/)

A schematic representation of the CFTR exon 9 mutants. In the minigene context, hCF contains several stop codons that disappear in F1 and F2, due to the indicated nucleotide deletions and insertions. Alternative splicing of the minigenes produces the CFTR exon 9− form, which is always in an open reading frame, and the CFTR exon 9+ form that may be in an open frame or not according to the mutations introduced in the exon. The bars represent the entire CFTR exon 9 and show the sequence variations and the positions of nonsense codons. The indicated nonsense codons apply only for the exonic sequences in the minigene. The asterisks indicate the two A to T substitutions in position 46 and 49, respectively. B, effect of cycloheximide on the hCF, F1, and F2 constructs. Hep3B cells were transfected with 3 μg of the minigene variants and treated with the indicated concentration of cycloheximide for 24 h as reported under “Experimental Procedures,” followed by radioactive RT-PCR amplification. Each point represents the mean of two independent experiments. C, autoradiography of exon 9+ and exon 9− splicing variants resolved on 6% polyacrylamide gel electrophoresis of actinomycin D-treated cells. Hep3B cells were transfected with hCF, F1, and F2 minigene variants, treated at the indicated time points with actinomycin D (5 μg/ml) as reported under “Experimental Procedures,” followed by radioactive RT-PCR amplification. The numbers below each lane represent the percentage of exon 9 inclusion expressed as the mean of two independent experiments done in duplicate. D, upper panel, autoradiography of exon 9+ and exon 9− splicing variants resolved on 6% polyacylamide gel electrophoresis. Hep3B cells were transfected with 3 μg of the indicated minigene variants, followed by radioactive RT-PCR amplification. In the lower panel, the RNA splicing variants detected by radioactive PCR were quantitated using a Cyclone. Each bar represents the mean ± S.D. of three independent experiments done in duplicate.
and out of frame cannot explain the different proportion of plus and minus forms observed.

Thus, the increase in exon recognition of the F1 and F2 constructs compared with hCF may be explained by the interference of the base insertions, deletions, and substitutions with exonic regulatory elements. To fully investigate the putative role of the stop codons in nonsense altered splicing, we introduced in the three hybrid minigenes two A to T substitutions in the central part of the exon (positions 46 and 49 of the exon, respectively) (Fig. 2A, Stop constructs). These nucleotide substitutions, according to the different reading frames of the hCF, F1, and F2 minigenes, do or do not generate stop codons. In the F1 and F2 contexts, these nucleotide changes generate two unique stop codons. The transfection experiments showed no changes in the splicing patterns with about 80% of exon inclusion (Fig. 2D, F1-Stop and F2-Stop). On the other hand, in the hCF minigene, the two A to T substitutions eliminated one stop codon, but again the splicing pattern was not affected, remaining at the initial 65% for this construct. This indicates that the two A to T substitutions, even if they produce nonsense codons, do not induce altered proportions of plus and minus forms, since they are not affecting splicing regulatory elements. We then tested, in the three different contexts, two of the natural substitutions with a splicing-inhibitory effect, the C31T and the C155A. In the hCF context, the C31T and the C155A do not introduce a new stop codon but reduce the 65% hCF exon inclusion to 48 and 16%, respectively (Fig. 2D, compare hCF with hCF-C31T and hCF-C155A). On the other hand, in the F1 and F2 minigenes, C31T creates a stop codon (Q414X), whereas C155A does not. The results shown in Fig. 2D indicate that these two mutations maintain a similar splicing-inhibitory effect both in F1 and F2 contexts. In fact, the 80% of exon inclusion in F1 and F2 is reduced in F1-C31T and F2-C31T to about 65%, and in F1-C155A and F2- C155A it is reduced to about 35% (Fig. 2D). These results indicate that the mutations have a negative effect on the exon recognition independently from the nonsense codon specification. In addition, we introduced on the F2 context two different stop codons in the same 5′-end position, with the creation of the F2-TAA and F2-TGA constructs. In one case, the stop codon (TAA) did not change the splicing pattern, whereas a 70% exon inclusion was observed for the TGA codon (Fig. 2D).

Altogether, these results indicate that substitutions introduced in the exon may affect the exon recognition, but this is completely independent from the fact that the substitutions do or do not create a stop codon. In conclusion, these data exclude the presence of a nuclear reading frame mechanism regulating CFTR exon 9 splicing and suggest that exonic mutations, including nonsense substitutions, cause changes in the splicing efficiency by affecting exonic splicing-controlling elements.

Identification of Regulatory Elements of Splicing in CFTR Exon 9—Three natural missense mutations with completely different effects on splicing (Q452P (A146C), which induces exon inclusion; A455E (C155A), causing exon exclusion; and V456F (G157T), with no effect) are located within 15 nucleotides. This suggests that this short sequence at the 3′ end of exon 9 may contain both enhancer and silencer functions, whose fine tuning on splicing could be missed if analyzed using multiple base deletions. To better characterize the mechanism by which some of the CFTR exon 9 mutations alter splicing, we performed a detailed investigation by site-directed mutagenesis of this short region (positions 144–157). This analysis was further extended to other point mutations distributed in the entire length of the exon (Fig. 3).

In the 15-bp sequence at the 3′ portion of the exon, the substitutions of wild type C in position 155, which corresponds to the base affected by A4555E, to either A (the natural mutation), G, or T induced exon skipping (Fig. 4, A and B). Interestingly, the 155G and 155T produced a similar percentage of exon inclusion of about 5%, which was the lowest among the different mutants. A reduction in the proportion of exon 9 inclusion was also observed as a result of point substitutions in the adjacent positions at 154 and 156 and for one of the mutants at position 153 (153T). On the contrary, the 153C and the 157T (V456F) variants did not significantly affect the splicing pattern. Extension of the mutagenesis in the 5′ direction, including the Q452P (146C) variant showed that mutants from position 145 to 149, with the notable exception of the 148G, induced exon inclusion (Fig. 4, A and B). The 148C substitution, in fact, contrary to the other nearby mutations, produced significant exon skipping. It is interesting to note that this particular mutation creates an overlapping and adjacent consensus sequence for 5′ and 3′ splice sites consisting of CAG-GTG. Finally, substitutions of wild type A in position 144 caused significant exon skipping (144T) or mild reduction in the percentage of exon inclusion (144G). These results indicate the presence of two juxtaposed silencer and enhancer elements in a short region of 15 nucleotides.

To study these elements in detail, we evaluated the effect on the splicing efficiency of double mutants. The 146C natural missense substitution (Q452P) with 95% of exon inclusion was analyzed in association with the nearby exon-skipping mutations in position 154 (C or T) and 155 (G or T). These two mutations produced a similar percentage of exon 9 inclusion of about 20% for 154C and 154T and 5% for 155G and 155T (Fig. 4C). Hybrid minigene experiments showed in three of the double mutants (146C-154C, 146C-154T, and 146C-155T) a percentage of exon 9 inclusion between 80 and 95% very similar to that found in 146C, indicating that the 146C enhancing mutation overrides the downstream silencing mutations. On the contrary, among the double mutants, only the 155G induced significant exon skipping when associated to the 146C enhancing variant (Fig. 4C). This indicates a strict functional interdependence of the juxtaposed silencer and enhancer elements related to the type of nucleotide substitutions. This points to a complex situation where these two functions may coexist in the same element as recently reported for the composite regulatory elements of splicing (CERES) in CFTR exon 12 (35).
It has been suggested that exonic splicing both enhancer and silencer functions.

Correlation between SR Protein Score Matrices and CFTR Exon 9 Splicing—It has been suggested that exonic splicing controlling elements affected by point mutations contain consensus sequences derived from SR-specific score matrices that mediate exon splicing efficiency (15, 18–20). We have determined the contribution of SR protein binding sites using the four available SF2/ASF, SC35, SRp40, and SRp55 motif-scoring matrices (15) to investigate wild type and mutant CFTR exon 9. The wild type CFTR exon 9 contains 15 multiple high score motifs (three for SF2/ASF, five for SC35, seven for SRp40, and no matches for SRp55) (Fig. 3). All of the mutants tested in the hybrid minigene experiments were analyzed according to the changes of the SR protein matrix scores (Table I). None of the natural missense mutations disrupts a preexisting SR protein site of this group, even if some do induce exon skipping. Interestingly, the majority of the mutations (10 of 15) inducing exon inclusion creates one or multiple new high score motifs, suggesting that the creation of new sites could be responsible for the increased exon usage. In the same manner, the majority of the mutants (10 of 15) that do not change the splicing pattern did not modify the SR protein scores. On the contrary, no correlation can be observed when the mutants induce exon skipping; in fact, among the 17 mutants inducing exon skipping, none disrupts an SR protein site. These results suggest that the currently available SR protein high score motif can explain only partially the complex regulation of CFTR exon 9 and that in this context any individual change in SR motif score cannot predict the splicing efficiency.

To directly evaluate the effect of one SR protein SF2/ASF, we cotransfected the WT exon 9 and mutant minigenes with SF2/ASF coding plasmid. The CFTR exon 9 is negatively regulated by SF2/ASF, and this inhibition is mediated by the interaction of the splicing factor with the intronic splicing silencer element (26). We reasoned that the creation or disruption of an SF2/ASF binding site in the exon should modify the response to SF2/ASF. For this reason, we studied the effect of SF2/ASF on several exonic mutants. The site-directed mutants analyzed include five substitutions that create a new SF2/ASF high score motif (A146G, G147C, T148C, T148G, and C155A) and two nucleotide substitutions that do not create new SF2/ASF sites (A20G and G154C) (Table I). In the absence of any cotransfected splicing factor and in comparison with normal exon 9, the mutations A20G, A146G, G147C, and T148C increase the percentage of exon inclusion, whereas T148G, G154C, and C155A induce exon skipping (Fig. 4 and Table I).

Cotransfection of SF2/ASF with the wild type CFTR exon 9 resulted in a decrease in exon 9+ transcripts as previously reported (26). In this case, the exon 9+ form goes from 65% to about 25% (Fig. 5). Cotransfection of SF2/ASF with each of the site-directed mutants showed in all cases a similar inhibitory effect of the splicing factor, with a significant reduction in the percentage of exon 9 inclusion (Fig. 5). In conclusion, cotransfection of SF2/ASF induced a significant reduction in the percentage of exon 9 inclusion not related to the SF2/ASF score motif variability (Fig. 5).

The Silencing Effect of the 155G Mutant Correlates with Specific hnRNPH Binding at the CFTR Exon 9—To identify specific changes in binding property or affinity of trans-acting factors at the juxtaposed enhancer and silencer elements that can explain the effect of the mutants, we performed UV-cross-linking experiments and pull-down assay on the 3′ portion of CFTR exon 9. In particular, we focused our attention on the two substitutions 155G and 155T, which, even if located at the same nucleotide position, were observed to induce exon skipping with different mechanisms. When alone, both caused severe exon skipping defects, but only 155G maintained a strong splicing inhibition when associated to the enhancing substitution 146C (Fig. 4C). UV-cross-linking experiments were done on mutants at position 155, on the 146C enhancing variant, on the 148A and 148G mutants, and on the double mutants 146C-155G and 146C-155T (Fig. 6). These mutant sequences and the WT were transcribed in vitro in the presence of [α-32P]UTP and equal quantities of labeled transcripts were then used in a UV-cross-linking assay with HeLa nuclear extracts. Among the numerous proteins that could be cross-linked to the labeled RNAs, a 55-kDa approximate molecular weight band could be observed only in the 155G and 146C-155G substitutions. No significant changes in the UV-cross-linked pattern compared with normal exon 9 were observed for the other mutants, which can be possibly ascribed to the fact that this experimental assay can detect predominant changes in binding properties of
abundant splicing factors. To identify the 55-kDa splicing factor binding at 155G, we performed pull-down experiments with WT exon 9, 155G, and 155A variants (Fig. 7A). Again, in the 155G mutant, a protein of about 55 kDa was evident from the proteins eluted from the beads that could not be pulled down by the WT or 155A sequence. Internal sequence analysis by mass spectrometry of the excised 55-kDa band yielded four different peptides and a search in the DDBJ/EMBL/GenBank data base revealed that their sequences were identical to residues 50–68, 99–101, 151–167, and 300–316 of hnRNPH. hnRNPH is an abundant splicing factor that in certain contexts binds to G-rich sequences, inducing splicing inhibition when the G residues are exonic (21–24). To investigate the binding properties toward hnRNPH of mutant RNAs, we performed Western blotting of the proteins eluted from the pull-down experiments. hnRNPH-specific immunoreactive material was present in the 155G mutant and in the double mutant 146C-155G, but not in 155A, 155T, and 146C-155T (Fig. 7B). A specific band for hnRNPH was also evident in the 164G+/H11001 silencing mutation, where a run of three G residues is formed by the insertion (Fig. 3), indicating that binding of these splicing factors is associated to the silencing effect. In conclusion, the 155G variant creates a new exonic splicing silencer element composed of five G residues, and presumably it is the specific binding of the hnRNPH inhibitory splicing factor that makes it dominant over the nearby enhancing mutations. These data suggest that the complex binding properties of the two juxtaposed elements modulates the splicing efficiency.

**DISCUSSION**

In this study, using the CFTR exon 9 as a model, we have addressed the function of exonic sequences in the correct exon recognition by the splicing machinery. We have analyzed the effect of premature termination codons, the role of splicing...

| WT sequence position | AA change | Nucleotide mutants | Exon 9+ | Disruption of preexisting sites | New sites created by the mutations |
|----------------------|-----------|--------------------|--------|-------------------------------|----------------------------------|
|                      |           |                    |        | SC35 | SR40 | SF2 | SR55 |
| WT                   |           |                    |        | 65  | 65  |     |     |
| A 15                 |           |                    |        |     |     |     |     |
| T 16                 | Δ         |                    |        |     |     |     |     |
| T 18                 | G         |                    |        |     |     |     |     |
| G 19                 | A         |                    |        |     |     |     |     |
| A 20                 | G         |                    |        |     |     |     |     |
| C 31                 | Q414X     |                    |        |     |     |     |     |
| A 43                 | G         |                    |        |     |     |     |     |
| A 44                 | N414S     |                    |        |     |     |     |     |
| 4649t                |           |                    |        |     |     |     |     |
| G 61                 | G424S     |                    |        |     |     |     |     |
| 66g57a69g            |           |                    |        |     |     |     |     |
| C 72                 | G         |                    |        |     |     |     |     |
| G 118                | D443Y     |                    |        |     |     |     |     |
| 120g122a123g         |           |                    |        |     |     |     |     |
| T 122                | I444S     |                    |        |     |     |     |     |
| A 144                | G         |                    |        |     |     |     |     |
| G 145                | G         |                    |        |     |     |     |     |
| A 146                | G         |                    |        |     |     |     |     |
| Q452P                |           |                    |        |     |     |     |     |
| G 147                | T         |                    |        |     |     |     |     |
| T 148                | G         |                    |        |     |     |     |     |
| T 149                | C         |                    |        |     |     |     |     |
| G 150                | A         |                    |        |     |     |     |     |
| T 151                | A         |                    |        |     |     |     |     |
| G 153                | C         |                    |        |     |     |     |     |
| G 154                | T         |                    |        |     |     |     |     |
| C 155                | A455E     |                    |        |     |     |     |     |
| G 156                | V456F     |                    |        |     |     |     |     |
| G 164+               | ins       |                    |        |     |     |     |     |

**TABLE I**

High score motifs were calculated according to Ref. 15. Amino acid (AA) changes are relative to the natural wild type context. In parentheses is indicated the starting nucleotide position of each SR protein binding.
regulatory sequences derived from SR-specific score matrices, and the creation of novel enhancer and silencer controlling elements. This extensive analysis has identified new elements with peculiar splicing regulatory properties that present juxtaposed enhancing and silencer functions.

To study the contribution of exonic sequences, we evaluated several naturally occurring missense and nonsense mutations and extended these studies by new variations introduced by site-directed mutagenesis. Our results show that single nucleotide substitutions may have a profound effect on the splicing efficiency inducing both exon inclusion and skipping. The large unexpected sensitivity to splicing variations (35 substitutions of 47 cause changes in the splicing pattern) may be related to the weak definition of the CFTR exon 9. In fact, the changes in the splicing pattern were modulated by the composition of the polymorphic TG-T locus in intron 8. This indicates the strict relationship between the definition of the exon mediated by the strength of the 3’ splice site and the exonic regulatory structures affected by the natural and site-directed mutants.

The existence of a putative nuclear scanning mechanism for nonsense codons that directly affects the splicing process, causing defective exon recognition, is controversial (1, 5–9, 15, 36–38). Using several hybrid minigene constructs, in which we systematically create and/or disrupt nonsense codons and reading frames, we showed that the creation of nonsense codons in exon 9 is not responsible per se in changes in splicing efficiency. In fact, exonic nucleotide substitutions may or may not modify the splicing pattern independently from the reading frame (Fig. 2). In addition, the treatment with protein synthesis or transcription inhibitors showed that the presence of multiple stop codons in exon 9 did not selectively affect the relative amount of one of the two mRNAs with or without exon 9. This indicates that selective degradation of the two spliced variants with stop codons is not responsible for changes in the measurement of splicing efficiency. Even if gene- and exon-specific determinants might be responsible for a selective exon splicing defect caused by nonsense codons, our results do not support the idea that the efficiency of exon recognition can be regulated by a putative nuclear reading frame scanning for nonsense codons. On the contrary, the fact that changes in splicing efficiency can be caused by a large number of point substitutions in
CFTR exon 9 strongly indicates that nucleotide variations induce changes in splicing efficiency by affecting exonic cis-acting regulatory elements. Our results are consistent with studies where the exon skipping phenotype has been shown to be reproduced also in in vitro splicing assay independent from the reading frame disruption (15, 20).

A significant result of this study is the lack of the predictive capacity of SR protein score matrices in CFTR exon 9 (Table 1). These matrices have been previously found to correlate with the splicing phenotype in other gene systems (15, 20). However, this is the first study to our knowledge that addresses the role of SR protein score matrices by extensive site-directed mutagenesis. Our analysis does not support a general predictive value of the SR matrices for the identification of CFTR exon 9 splicing regulation.

The limit of detection may be below that of UV-cross-linking and pull-down assays used in this paper. Alternatively, each mutation may modulate the cooperative propagation of splicing factors along the exon as previously suggested for hnRNPA1 in the human immunodeficiency virus-1 tat exon 3 (40). It is interesting to note that 144T, 155T, and most significantly 148G cause a variable degree of exon skipping and create an AG-CT sequence, which might represent a consensus for both 5’ and 3’ splice sites. In some cases, sequences with inhibitory activity have been shown to function as pseudosilencers, which sequester the splicing machinery into a nonproductive complex (41–43). Inappropriate exon skipping may also result from the creation of U1 small nuclear ribonucleoprotein binding sites (30). Not only creation, deletion, or changes in binding affinity of binding sites for splicing factors but also changes in secondary structure, having an indirect effect on such interactions, might participate in the regulation of the splicing efficiency, modulating the proper display of splicing factor binding sequences (44).

Point mutations in exonic splicing regulatory elements may have pathological consequences. CFTR exon 9 skipping has been associated with CF phenotypes of different severity and produces a nonfunctional CFTR protein (45). Some of the missense mutations analyzed in this study may exert part of their phenotypic expression and disease variability affecting the splicing pattern, in particular when associated to an unfavorable polymorphic variant near the 3’ splice site. For example, it appears that A455E can achieve adequate levels of chloride conduction at the cell surface (46, 47), causing only a partial CFTR protein processing defect (48). Since it also increases exon skipping, the penetration of its pathological effects may be better related to the deletion of the amino acids encoded by the entire exon 9. Furthermore, the modulation by the concentration of splicing factors, which have an inhibitory effect on the CFTR exon 9 (26, 49) and a specific and possibly individual variation distribution, can provide an explanation for the phenotypic and tissue-specific variability in CF patients, particularly in those carrying the A455E substitution. It is suggestive that as many as five natural mutants in the CFTR exon 9 modify the splicing pattern with either an increase or decrease in the splicing efficiency (Fig. 1A). It is also interesting to note that mutants inducing aberrant exon skipping may also occur at the third position of the codon usage and do not change the amino acid code. When these mutations are found in genomic screening, their effect on splicing may not be taken into account, a fact that would lead to an erroneous molecular diagnosis. Thus, medical geneticists should be aware that silent mutations cannot be ignored as a potential cause of disease.

The extreme sensitivity of exonic sequences to splicing deregulation is of great relevance to human pathology, since missense and even translation silent variations may cause human disease by affecting the extent and accuracy of pre-mRNA splicing.

Acknowledgments—We thank Adrian Krainer for the SR protein score motifs, Thilo Doerk for helpful discussion, and Ann Crum for proofreading the manuscript.

REFERENCES

1. Dietz, H. C., Valle, D., Francomano, C. A., Kendzierski, R. J., Jr., Pyeritz, R. E., and Cutting, G. R. (1993) Science 259, 680–683
2. Shiga, N., Takeshima, Y., Nakamoto, M., Inoue, K., Yokota, Y., Yokoyama, M., and Matsuo, M. (1997) J. Clin. Invest. 100, 2204–2210
3. Larson, C. L., Hahnen, E., Androphy, E. J., and Wirth, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6307–6311
4. D’Souza, I., Pookaj, P., Hong, M., Nocchi, D., Lee, V. M., Bird, T. D., and Schellenberg, G. D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5588–5603
5. Dietz, H. C., and Kendzierski, R. J., Jr. (1994) Nat. Genet. 8, 183–188
6. Aoudjouchi, S., Velamos, J., and Mistele, C. (1996) Cell 85, 415–422
7. Lorenzo, F., Maertzdorf, B., Pannell, R., and Milstein, C. (1994) EMBO J. 13, 4617–4622
Missense, Nonsense, and Neutral Mutations Define Juxtaposed Regulatory Elements of Splicing in Cystic Fibrosis Transmembrane Regulator Exon 9
Franco Pagani, Emanuele Buratti, Cristiana Stuani and Francisco E. Baralle

J. Biol. Chem. 2003, 278:26580-26588.
doi: 10.1074/jbc.M212813200 originally published online May 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212813200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 28 of which can be accessed free at http://www.jbc.org/content/278/29/26580.full.html#ref-list-1