SR protein-mediated inhibition of CFTR exon 9 inclusion: molecular characterization of the intronic splicing silencer

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ABSTRACT

The intronic splicing silencer (ISS) of CFTR exon 9 promotes exclusion of this exon from the mature mRNA. This negative influence has important consequences with regards to human pathologic events, as lack of exon 9 correlates well with the occurrence of monosymptomatic and full forms of CF disease. We have previously shown that the ISS element interacts with members of the SR protein family. In this work, we now provide the identification of SF2/ASF and SRp40 as the specific SR proteins binding to this element and map their precise binding sites in IVS9. We have also performed a functional analysis of the ISS element using a variety of unrelated SR-binding sequences and different splicing systems. Our results suggest that SR proteins mediate CFTR exon 9 exclusion by providing a ‘decoy’ sequence in the vicinity of its suboptimal donor site. The results of this study give an insight on intron ‘exonization’ mechanisms and provide useful indications for the development of novel therapeutic strategies aimed at the recovery of exon inclusion.

INTRODUCTION

The regulation of CFTR exon 9 splicing has been extensively studied in recent years because of its clear connection with CF disease (1–6). At present, several splicing controlling regions have been characterized near the 3’ and 5’ boundaries of this exon. These include a polymorphic TG(m)T(n) region near the 3’ss, a suboptimal donor site and a Polypyrimidine-rich Controlling Element just downstream of the 5’ss (PCE) (7–9). Moreover, additional controlling regions have been identified inside the exon itself in the form of CERES elements (10) and as an intronic splicing silencer region (ISS) further away in the IVS9 intron sequence (11) (Figure 1A). During the course of these studies several trans-acting elements have also been identified as binding specifically to these regulatory elements: TDP-43 to the (TG)m region near the 3’ss of the exon, which has been recently shown to recruit hnRNP proteins near the 3’ss (6,12,13), TIA-1 to the PCE that promotes exon inclusion (9), and unidentified members of the SR protein family to the ISS sequence (11) (Figure 1B).

The SR protein family (14–16) has been predominantly studied in relationship with its involvement in alternative and constitutive splicing control (17–22) and indeed may have played a decisive role in the evolution of this process (23,24). However, it has also been recently shown to participate in a very wide range of functions that include the maintenance of genomic stability (25–27), mRNA export (28–31), mRNA surveillance (32) and protein translation (33,34).

In splicing regulation, SR proteins are generally considered to bind exonic splicing enhancer (ESE) sequences (35–37) and in this way they generally promote exon inclusion in the pre-mRNA molecule that is processed by the spliceosome. This enhancement is achieved in a variety of ways: by antagonizing the effect of negative regulators such as hnRNP proteins (38–41), by directly recruiting basic splicing factors such as U1 and U2snRNPs to the exon acceptor and donor sites (42,43), and by promoting spliceosome assembly through their RS domains (20,21). Because of all these functions, SR proteins represent one of the most important factors that promote exon inclusion (44) and is not surprising that an excess of SR proteins can compensate for complete U1snRNP inactivation and rescue correct splicing (45,46).

In general, most SR proteins share rather common enhancer properties despite they have different sequence binding abilities (47), protein domain compositional differences (15) or nucleo cytoplasm shuttling properties (48). However, this is by no means a rule. In fact, some SR protein family members have also been recently identified in connection with splicing repression. For example, a novel SR protein designated SRp38 has been recently demonstrated to posses splicing inhibitory activity in mitotic cells or following heat shock treatment (49–51).
In addition, another SR-protein like factor (SR-15) has recently been described to possess general splicing inhibitory activity in the HSV1 virus (52).

Most importantly, there are many examples of factors that display either enhancer or repression activity in one system can display the opposite behavior in different pre-mRNAs. The SR proteins family is no exception to this observation. Indeed, past research has led to the discovery of a small number of splicing systems in which normally enhancing SR proteins display a inhibitory activity on the splicing process (53–60).

As previously mentioned, to this short list of examples we have to add the reports that describe SR proteins as general inhibitors of CFTR exon 9 splicing (11,61). To this date, however, no clear identification/mapping or functional binding sites for these inhibitory SR proteins has been provided. In this work, we have aimed to cover this gap and investigate the functional reasons that underlie this particular SR inhibitory activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid construction**

Plasmid TG11T5 has been previously described by Niksic et al. (7). Plasmid pES was obtained by deleting part of the original IVS9 sequence in TG11T5 and inserting in its place a PstI/KpnI linker just before the NdeI cloning site (Figures 2A and 3A). All the other plasmids used in this study were obtained by cloning the sequence of interest in the pES plasmid either in the PstI/KpnI (PK series) or PstI site (P series). This was achieved by annealing two complementary oligonucleotides containing the sequence of interest and ligating according to standard protocols (sequence of the oligonucleotides is available upon request). In order to mutate the cryptic 3’ss sequence in the TG11T5 context (mutant IVS9del3’ss) we used the two following oligos: 5’ctcttttttttttctaaatttgtagtg3’ and 5’cactcaaaatagaaagaaaagag3’ antisense.

**In vitro transcription, UV cross-linking and immunoprecipitation analysis**

To generate the RNA probe of h3’int a corresponding pBluescript II KS plasmid containing this sequence was linearized with NdeI and transcribed with T7 RNA Polymerase (Pharmacia Biotech) in the presence of α32P-UTP, according to standard procedures. The UV cross-linking assay was performed by incubating 1 x 106 c.p.m.-labeled RNA probes with 100 μg of total HeLa nuclear extracts (CilBiotech, Mons, Belgium) and 100 μg heparin in a 20-μl final reaction volume containing 20 mM HEPES pH = 7.9, 72 mM KCl, 1.5 mM MgCl2.

**Figure 1.** (A) Shows a schematic diagram of the splicing controlling regions of CFTR exon 9, both within the exon (CERES element) and in the flanking IVS8 and IVS9 sequences: TG(m)T(n), PCE and ISS. h3’int defines the IVS9 region that includes both the PCE and ISS controlling elements. (B) Shows the trans-acting factors identified up to now that bind to these elements. (C) Shows an immunoprecipitation analysis of the h3’int region and of the fibronectin EDA ESE element both in its wild-type (hTot) and mutated form (h2e). The left, central and right panels show the immunoprecipitation profiles obtained from each RNA using mAb 96 (specific against SF2/ASF), mAb 1H4 (specific against the phosphorylated RS domain) and an anti-SC35 antibody.

**Figure 2.** (A) Shows the sequence of the entire IVS9 (h3’int) sequence from the last nucleotides of exon 9 (in capital letters) to the NdeI site that was cloned in the TG11T5 original minigene. Also shown in this diagram are the positions of the two mutated nucleotides that create a unique PstI site. In the ISS region, the sequences targeted by each of the four antisense oligos (AS1–AS4) are underlined. (B) and (C) shows the immunoprecipitation profiles of the h3’int sequence using mAb 96 (B) and mAb 1H4 (C) monoclonal antibodies in the presence of each of the four antisense oligos. The migration of SF2/ASF and of SRp40 are marked with an arrow.
0.78 mM magnesium acetate, 0.52 mM dithiothreitol, 3.8% glycerol, 0.75 mM ATP and 1 mM GTP for 15 min at 30°C. Samples were transferred to HLA plate (Nunc, InterMed) on ice and irradiated with 0.8 J UV light for 5 min by using a BIO-LINK apparatus (Euroclone). Unbound RNA was digested with 30 μg of RNase A (Sigma) for 30 min at 37°C and incubated for 2 h at 4°C on a rotator wheel with 150 ml of IP buffer and then loaded onto a SDS-10% PAGE gel. Gels were run at a constant 30 mA for ~3.5 h, dried under vacuum, and exposed for 4 days with a BioMax Screen (Kodak).

Transient transfection minigene splicing analysis in Hep3B cells

Liposome-mediated transfections of 3 x 10^5 human hepatocarcinoma Hep3B cells were performed using DOTAP Liposomal Transfection Reagent (Alexis Biochemicals) according to manufacturer instructions. After 18 h the transfection medium was replaced with fresh medium and 24 h later the cells were washed with PBS and RNA was purified using RNAwiz (Ambion). RT–PCR reactions to specifically amplify the minigene transcripts was performed as previously reported (11). In order to quantify the amplified fragments, the PCR reaction was performed in the presence of α^32P-UTP and the samples run on a 5% denaturing polyacrylamide gel. Radioactive intensity was measured using a Cyclone (Packard). Transfection of a siRNA reagent against TDP-43 were performed as previously published (62).

RESULTS

Characterization of SR proteins binding to the ISS element in IVS9

In order to better define which SR proteins are binding to the ISS region we performed immunoprecipitation analysis using, as substrate, the entire h^3'int intronic region (Figure 1A). Figure 1C shows an immunoprecipitation analysis with HeLa nuclear extract of this RNA (h^3'int) together with two control RNAs from the fibronectin EDA exon, one bearing a well-characterized ESE sequence (hTot) and one where this sequence has been deleted (h^3'e) (63). Each RNA was labeled using α^32P-UTP and incubated with 150 μg of Hela nuclear extract before being subjected to UV-crosslinking and digestion with RNAse A. Samples were then run on a 10% SDS–PAGE gel and exposed using BioMax autoradiographic films. Immunoprecipitation was performed using equal amounts of each UV-crosslinked sample and following the addition of specific monoclonal antibodies against SF2/ASF (Figure 1C, left panel, mAb 96), anti-RS phosphorylated domain (mAb 1H4) (Zymed Laboratories Inc) and an anti-SC35 monoclonal antibody (Sigma). The mobility of the SR proteins is indicated by arrows. The results show that SF2/ASF and SRp40 are the major SR protein family members binding to the h^3'int region.
Mapping of the SR protein-binding regions in h3'int using small antisense oligos

In order to map the binding sites of each SR protein in h3’int we used in immunoprecipitation analysis a set of antisense oligos which targeted the original ISS region (Figure 2A, AS1–AS4). Figure 2A shows a schematic diagram of the entire IVS9 sequence inserted in the original TG11T5 plasmid (7) together with the sequences targeted by the AS1–AS4 antisense oligos that cover the originally mapped ISS region (11). Also shown in this figure are the two single-point mutations introduced in h3’int to create a unique PstI cloning site that can be used to remove this entire region from the template minigene plasmid. Based on the results obtained in Figure 1C, each oligo was then used in immunoprecipitation analysis with mAb 96 and mAb 1H4 together with the h3’int labeled RNA. As shown in Figure 2B lane 3, oligo AS2 was the most efficient in inhibiting SF2/ASF binding to h3’int whilst oligo AS1 was very efficient in blocking SRp40 binding to this region (Figure 2C, lane 2). A lesser amount of inhibition for SRp40 binding could also be detected in the presence of the AS4 antisense oligo (Figure 2C, lane 5). However, as the AS1 and AS4 sequence share considerable similarity (almost 50%, especially in the ‘agaaat’ central region) the AS4 oligo may have cross-hybridized with the AS1 sequence blocking partially its interaction with SRp40 in vitro. This hypothesis is consistent with the observed lack of functional effects of the AS4 sequence alone on CFTR exon 9 inclusion.

Functional importance of the different AS1 to AS4 sequences on CFTR exon 9 inhibition

Figure 3A shows a schematic representation of the CFTR exon 9 hybrid minigene construct lacking the ISS sequence (pES). In this construct, the IVS9 sequence was shortened by exploiting the creation of a novel PstI site which was directly joined to NdeI through a small linker that also provided a unique KpnI site. The unique PstI/KpnI sites could then be used to insert different combination of the AS1–AS4 sequences in the ISS position (Figure 3A, lower panel, has a detailed scheme of the inserted fragment position in the pES plasmid). Based on the results of the immunoprecipitation analyses it was then decided to insert in pES the two combinations of AS1 + AS2 (pTB AS1 + AS2PK) and AS3 + AS4 (pTB AS3 + AS4PK). Figure 3B shows the RT–PCR assays of these plasmids (lane 3 and lane 4, respectively) following transfection in Hep3B cells together with two control minigene constructs: the original TG11T5 (lane 1) and the pES minigene (lane 2). The upper and lower bands correspond to exon 9 inclusion (ex9+) and exclusion (ex9−), respectively. Quantification of these bands from three independent experiments following radioactive RT–PCR (Figure 3C) demonstrated that the SR-binding AS1 + AS2 region could entirely recover the ISS inhibitory activity displayed by the original TG11T5 minigene construct (compare lanes 1 and 3). On the other hand, the AS3 + AS4 region that had no apparent SR-binding ability could not display any inhibitory activity with respect to the original pES plasmid (compare lanes 2 and 4).

A similar pattern could also be observed when each individual sequence was inserted in the pES plasmid (Figure 3D). A quantitation of the inhibitory activity of each sequence (Figure 3E) confirmed that only the AS1 and AS2 sequences (Figure 3D, lanes 2 and 3) could inhibit CFTR exon 9 inclusion whilst AS3 and AS4 did not cause any drop in CFTR exon 9 splicing efficiency (Figure 3D, lanes 4 and 5). Moreover, the fact that the AS1 and AS4 showed very distinct inhibitory activities supports the conclusions drawn from the immunoprecipitation experiments in Figure 2C.

Inhibitory effects of other SR-binding sequences on CFTR exon 9 inhibition

In consideration of the fact that ISS activity strongly correlates with AS1 + AS2 it was of interest to determine whether different SR protein-binding sequences were also capable of inhibiting CFTR exon 9 inclusion. To this end, it was decided to test the activity of two unrelated SR-binding sequences that have been extensively characterized by our laboratory: the ESE region of the fibronectin EDA exon (63) and the ISE region (ApoISE) of the ApoAII intron 3 (64) (Figure 4A). The advantage of using these sequences is represented by the fact that they all possess different combinations of SR protein-binding abilities (Figure 4A) and also that in their respective contexts they functionally behave as ESE and ISE elements, respectively. These additional polypurinic sequences were then cloned in the PstI and KpnI sites of the pES plasmid and transfected in Hep3B cells. Figure 4B and C show that both the EDA ESE sequence (pTB-EDA PK, lane 1), and the ApoISE sequence (pTB ApoI SEP K, lane 2) were capable of inhibiting CFTR exon 9 inclusion in a way comparable with that observed with pTB AS1 + AS2PK (lane 3) and higher than the pES plasmid alone (lane 4).

For the sequences that can bind SF2/ASF, the functional specificity of SR protein binding on the inhibitory activity was also investigated by overexpressing this particular SR protein in transfected cells (Figure 4D). As shown in this figure, the increased levels of CFTR exon 9 inhibition for constructs pTB AS1 + AS2PK (Figure 4D, lanes 3 and 4) and pTB-EDA PK (Figure 4D, lanes 5 and 6) is comparable to that observed for the TG11T5 plasmid (Figure 4D, lanes 1 and 2) and greater than the one observed for the pES plasmid alone (Figure 4D lanes 7 and 8). In addition, SC35 overexpression does not result in increased levels of CFTR exon 9 skipping in the pTB AS1 + AS2PK plasmid (Figure 4D, lanes 9 and 10). This result is consistent with the immunoprecipitation results shown in Figure 1. We have also tried overexpression of SRp55 and SRp75 that also induced CFTR exon 9 skipping to different degrees (data not shown), in keeping with the SR protein response profile obtained in the original study by Pagani et al. (11). The binding experiments did not show reproducible direct interaction of these proteins with h3’int, although at times bands compatible with SRp55 and SRp75 were visible (Figure 1C, middle...
expression, following transfection in Hep3B cells.

The response of the pTB AS1 + AS2PK construct to SC35 overexpression, and of (in vitro) (Figure S1). This is a well-known context. To this end, we cloned the AS1 + AS2 and were capable of acting as ESEs in a heterologous splicing exon 9 AS1 + AS2 ISS sequence and the Apo ISE sequence acting as ISS elements in the CFTR exon 9 context.

Therefore, it was of interest to determine whether the AS1 + AS2 sequence contains all the functional properties of the originally mapped ISS element. The results in Figure 4 conclusively show that both the EDA ESE and the Apo AII ISE elements. The column on the right shows the SR protein-binding specificity of all these sequences as previously determined by immunoprecipitation analysis. The EDA ESE and Apo ISE sequences were then inserted in the pES plasmid (as shown in the lower panel), thus creating constructs pTB EDAPK and pTB ApoISEPK, respectively. The levels of CETR exon 9 inclusion displayed by these three plasmids in Hep3B cells and compared with that of the pES and pTB AS1 + AS2PK constructs is reported in (B).

A quantification of three independent experiments is reported in (C). (D) Shows the response of the TG11T5, pTB AS1 + AS2PK, pTB EDAPK and pES plasmids to SF2/ASF overexpression, and of the response of the pTB AS1 + AS2PK construct to SC35 overexpression, following transfection in Hep3B cells.

The lack of correlation between this absence of binding in IP assays and functional experiments may be due to the well-known non-specific effects of SR protein overexpression or to indirect interactions with the ISS sequence. Further studies will be needed to clarify these differences. In any case, taken together, these data further reinforce our conclusion that the AS1 + AS2 sequence is a much more efficient ESE (66%) than the AS1 + AS2 sequence (20%).

These results also suggested that the action of the ISS element could be dependent on the presence of the naturally occurring cryptic 3‘ss in CFTR IVS9, perhaps through the recruitment of a non-productive spliceosomal complex in this position. However, mutating this 3‘ss sequence from ‘ag’ to ‘aa’ in the natural TG11T5 context (mutant IVS9del3‘ss) did not significantly affect the efficiency of CFTR exon 9 inclusion (Figure 5C). This result ruled out the possibility that this acceptor-like sequence alone could be playing a role in wild-type ISS functioning.

Improving acceptor site recognition of CFTR exon 9 results in context-specific effects on the splicing pattern

To better assess the context-dependent effects of these different SR protein-binding sequences in the vicinity of CFTR exon 9 we then improved the definition of this exon by removing TDP-43 through siRNA treatment (Figure S2). As previously demonstrated, TDP-43 is a major inhibitory splicing factor that specifically recognizes the TGm polymorphic locus in IVS8 (6,12), and its specific removal from the transfected cell can offset many splicing inhibitory effects, including those mediated by the ISS (62). It was therefore interesting to test the effect of removing this factor on the splicing patterns of the pTB AS1 + AS2PK, pTB ApoISEPK and pTB EDAP PK minigenes. Figure S2 shows that removal of TDP-43 in these different

Changing ISS/ISE to ESE activities in the pES context by providing a heterologous donor site

In order to further test these potential enhancer activities of AS1 + AS2 and ApoISE in the CFTR exon 9 context it was then decided to insert in the pES plasmid a donor sequence that could be used as a viable 5‘ss to promote ‘exon’ inclusion. This was achieved by simply cloning the AS1 + AS2, ApoISE, and EDA ESE sequences in the pES plasmid. Figure 5A shows that this cloning procedure, as opposed to cloning in the PstI/KpnI sites, provided any PstI-inserted sequence with a downstream donor site sequence possessing a score of 0.76 according to the NNSPLICE predictor program (67). The results of this cloning procedure on the resulting pTB AS1 + AS2PK, pTB ApoISEPK and pTB EDAPPK plasmids are reported in Figure 5B, lanes 1, 3 and 5, respectively. In all three cases, the cloning in the PstI site alone resulted in the appearance of an extra band which, when sequenced, was shown to consist in a ‘mini-exon’ sequence that exploited the newly inserted 5‘ss and a cryptic 3‘ss in the h3‘int sequence (Figure 5B, lower panel). Interestingly, the intensity of this extra band in the pTB AS1 + AS2PK and pTB ApoISEPK is markedly different, with the pTB AS1 + AS2PK splicing profile still retaining some of the ex9+/ex9- splicing forms. This is consistent with the different ESE abilities displayed in the dsx-XH plasmid (Figure S1), where the ApoISE sequence is a much more efficient ESE (66%) than the AS1 + AS2 sequence (20%).

Figure 4. (A) Upper panel shows a comparison between the AS1 + AS2 sequence and the heterologous polyurinic sequences from the EDA ESE and the Apo AII ISE elements of the Tg11T5, pES, pTB AS1 + AS2PK, pTB ApoISEPK, and pTB EDAPPK constructs. The levels of CFTR exon 9 inclusion displayed by these three plasmids in Hep3B cells and compared with that of the pES and pTB AS1 + AS2PK constructs is reported in (B). A quantification of three independent experiments is reported in (C). (D) Shows the response of the TG11T5, pTB AS1 + AS2PK, pTB EDAPK and pES plasmids to SF2/ASF overexpression, and of the response of the pTB AS1 + AS2PK construct to SC35 overexpression, following transfection in Hep3B cells.

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which processing of the IVS3 intron from the Drosophila doublesex (dsx) gene is dependent on the sequences inserted at the 3’ end of the construct. As shown in Figure S1, both the AS1 + AS2 and ApoISE sequence display higher ESE activity than the control AS3 + AS4 sequence.
contexts can have very different outcomes depending on the type of enhancer sequence present in the ISS position.

Inhibitory effects of a C/A-rich YB-1 binding sequence in the ISS position

Finally, it was interesting to assess whether the inhibitory effects mediated by these different G/A-rich sequences in the ISS position could also be mimicked by a non-polypurinic sequence with well-known splicing enhancer effects. Therefore, an A/C-rich enhancer sequence from the alternatively spliced exon v4 of the CD44 gene was inserted in the ISS position (Figure 6A) (68). This sequence was identified by Stickeler et al. (68) as the binding site of YB-1, a member of the family of multifunctional cold shock domain proteins (CSD proteins). As shown in Figure 6B, cloning this YB-1 sequence only in the PstI site of the pES plasmid (pTB YB-1P) as opposed to the PstI/KpnI sites (pTB YB1PK) is shown in (C). The schematic diagram on the left shows the composition of the unique splice product observed in lane 1 whilst the position of the transcripts including exon 9 (ex9+) and lacking exon 9 (ex9−) are marked on the right.

Figure 5. (A) Shows a comparison of the different splice site composition when the AS1 + AS2, EDA and ApoISE sequences are cloned in the PstI/KpnI sites (upper panel, PK plasmid series) as opposed to the PstI site alone (lower panel, P plasmid series). The differing splice site composition in the region surrounding the point of insertion is indicated by arrows, with the conserved 3′ss ‘ag’ and 5′ss ‘gt’ nucleotides highlighted in bold. (B) Shows a comparison of the splicing profiles between P and PK plasmids carrying the AS1 + AS2, EDA and ApoISE sequence following transfection in Hep3B cells. The asterisk shows the extra band that is observed in the plasmids carrying the heterologous donor site downstream of the inserted sequence (pTB AS1 + AS2P, pTB EDAP and pTB ApoISEP). The lower panel shows a schematic diagram of the new splicing event indicated by the asterisk whilst the position of the transcripts including exon 9 (ex9+) and lacking exon 9 (ex9−) are marked on the right. (C) Left panel shows a transfection analysis of a TG11T5 mutant (IVS9del30) carrying a ‘ag’ to ‘aa’ mutation in the cryptic 3′ss sequence. The CFTR exon 9 inclusion levels are comparable to those detected for the TG11T5 wild-type plasmid (C, right panel).

Figure 6. (A) Shows the sequence of the YB-1-binding motif inserted in the PstI/KpnI restriction sites of the pES plasmid (pTB YB1PK). (B) Left panel shows a comparison of the levels of CFTR exon 9 inclusion in the TG11T5, pES and pTB YB1PK plasmids following transfection in Hep3B cells. The position of the transcripts including exon 9 (ex9+) and lacking exon 9 (ex9−) are marked on the right. The results of three independent experiments as quantified by radioactive RT–PCR are reported in (B), right panel. The effect of cloning the YB-1 sequence only in the PstI site of the pES plasmid (pTB YB-1P) as opposed to the PstI/KpnI sites (pTB YB1PK) is shown in (C). The schematic diagram on the left shows the composition of the unique splice product observed in lane 1 whilst the position of the transcripts including exon 9 (ex9+) and lacking exon 9 (ex9−) are marked on the right.
DISCUSSION

CFTR exon 9 splicing is a complex event in which several cis-acting elements located in both intronic and exonic sequences play an important role (6–8,10). In particular, an ISS sequence in IVS9 has been previously shown by Pagani et al. (11) to represent a negative element towards its inclusion in the final mRNA molecule.

Our work has shown that this region specifically binds two members of the SR protein family, SF2/ASF and SRp40, which are normally associated with binding to enhancer elements that promote exon inclusion. Because of this, the CFTR ISS is one of the relatively few splicing systems in which SR proteins behave as suppressors of splicing. It is therefore interesting to compare its functioning with other systems in which SR proteins have also been identified as negative splicing regulators (53–60) (Figure 7).

In the hnRNP A1 gene ISS, a particular SR protein (SRp30c) can recognize the silencer element (CE9) and directly down-regulate exon recognition (53) (Figure 7A). Most importantly, CE9 cannot compromise the assembly of U2-dependent complexes on the 3'ss of hnRNP A1 exon 8 (69) and cannot function as an enhancer element in a heterologous system (53). Although the molecular mechanism of CE9 functioning remains unclear, this represents an important functional difference with respect to the CFTR exon 9 ISS, because the AS1 + AS2 sequence can function as an enhancer sequence in a heterologous splicing system.

A second type of inhibition has also been found in the case of what is really a ISE element localized downstream of b-tropomyosin gene exon 6A (57). In this case, competition occurs between a SR protein enhancer factor (SF2/ASF) and another SR protein that has no enhancer effect in this system (SC35) (Figure 7B). Also this model does not seem to apply to the CFTR exon 9 ISS working model for a number of reasons. First of all, mapping of the SF2/ASF and SRp40-binding sites on the CFTR ISS sequence has shown that they are physically distinct, making competition between the two highly unlikely (unlike the case of the ISE element of b-tropomyosin exon 6A where the binding sites of SF2/ASF and SC35 actually overlap). In addition, our observation that heterologous SR-binding sequences with rather different splicing specificities (EDA ESE and Apo ISE elements) and even a YB-1 binding sequence can restore CFTR ISS function in the absence of its natural sequence would tend to rule out any specific effects by particular SR proteins.

A higher degree of similarity can be found between CFTR exon 9 ISS and the action of the IIIa repressor element (3RE) originally described in Adenovirus (58) (Figure 7C). In fact, just like CFTR exon 9 ISS, also the 3RE sequence was observed to function as an enhancer element when inserted in a heterologous splicing system, showing that the mechanisms of action of 3RE is wholly dependent on context. From a functional point of view, the inhibitory mechanism mediated by 3RE was initially thought to reside in the physical inhibition of U2snRNP binding to the IIIa 3’ss, because of its nearness to the IIIa acceptor site (30 nt) (58). More recently, the inhibitory activity of SF2/ASF on IIIa splicing has been specifically identified as residing in its second RNA binding domain, RBD2, although the exact mechanism still remains to be defined (70). It is difficult to determine whether physical hindrance may also represent the mode of action for CFTR ISS as this sequence is localized rather far away from the CFTR exon 9 5’ss (80–100 nt). Given this limitation, the potential physical hindrance between U1snRNP binding to the natural donor site and SF2/ASF and SRp40 binding to AS1 + AS2 seems unlikely.

Another SR-mediated inhibitory situation is represented by the Negative Regulator of Splicing (NRS) of the Rous Sarcoma Virus (Figure 7D). In this case, together with SR protein SF2/ASF (71), the NRS can also bind a U1snRNP molecule (60), a U11 snRNP molecule (72) and hnRNP H (73). Interestingly, also this sequence can function as an enhancer element when inserted in the dsx-HX in vitro splicing system (59). However, the mechanism of action of the NRS sequence...
cannot be explained by physical hindrance because it is localized very far away from the 3'ss that is inhibited. Recent research has shown that the exact mechanism through which splicing inhibition occurs may probably reside in the formation of non-productive complexes between the NRS inhibitory splicing complex, the 3'ss of the src exon, and the polyadenylation process (60,74). In addition, it has been suggested that RNA polymerase II can ‘tether’ emerging splice sites in the pre-mRNA (75). In this case the NRS might also act as a disturbing presence for the recognition of the natural 5'ss.

The CFTR exon 9 ISS is an excellent example of the importance of sequence-context in determining the action of cis-acting sequences (76). In the natural situation, the heterologous 5'ss sequence is absent hence no IVS9 intron sequences may be ‘exonized’. Nonetheless, the presence of the exon enhancer-like complexes formed by SR proteins or YB-1 in the ISS may create a situation in which the U1snRNP molecule approaching CFTR exon 9 would remain ‘undecided’ between binding to the wild-type suboptimal site and waiting for an indication from the SR/YB-1 proteins present in the ISS of a better target immediately downstream (Figure 7E). This is consistent with the fact that the outcome of TDP 43 removal depends on the strength of the ISS–SR interactions. Weak SR interactions and removal of TDP 43 lead to complete recovery of CFTR exon 9 inclusion while strong SR interaction results in the inclusion of a super-exon 9 sequence by selecting exclusively the new 5'ss downstream of the ISS (see Fig. S2). This data suggests that the ISS may act as a sort of ‘decoy’ system hampering recognition of the exon 9 5'ss. The result of this stalemate would be a net decrease in CFTR exon 9 donor site recognition and, consequently, in a lesser inclusion of CFTR exon 9 in the mature mRNA. In this respect, therefore, a critical issue might be represented by the processing speed of the RNA polymerase II molecule in presenting the ISS sequence after having transcribed the CFTR exon 9 region, and these issues are currently being investigated in our laboratory.

Finally, from a pathological point of view, the importance of having mapped exactly the binding sites for the SR trans-acting factors that are responsible for this inhibitory action can provide researchers with a useful target to inhibit their action, for example, by antisense oligonucleotide approaches (77,78).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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