REPORT

Cryptic splice site usage in exon 7 of the human fibrinogen B β -chain gene is regulated by a naturally silent SF2/ASF binding site within this exon

SILVIA SPENA,¹ MARIA LUISA TENCHINI,¹ and EMANUELE BURATTI²

¹Department of Biology and Genetics for Medical Sciences, University of Milan, 20133 Milan, Italy

²International Centre for Genetic Engineering and Biotechnology (ICGEB), 34012 Trieste, Italy

ABSTRACT

In this work we report the identification of a strong SF2/ASF binding site within exon 7 of the human fibrinogen B β -chain gene (*FGB*). Its disruption in the wild-type context has no effect on exon recognition. However, when the mutation IVS7 + 1G>T—initially described in a patient suffering from congenital afibrinogenemia—is present, this SF2/ASF binding site is critical for cryptic 5'ss (splice site) definition. These findings, besides confirming and extending previous results regarding the effect of SF2/ASF on cryptic splice site activation, identify for the first time an enhancer sequence in the *FGB* gene specific for cryptic splice site usage. Taken together, they suggest the existence of a splicing-regulatory network that is normally silent in the *FGB* natural splicing environment but which can nonetheless influence splicing decisions when local contexts allow. On a more general note, our conclusions have implications for the evolution of alternative splicing processes and for the development of methods to control aberrant splicing in the context of disease-causing mutations.

Keywords: cryptic splice sites; *FGB* gene; ESE; SF2/ASF protein

INTRODUCTION

During the splicing pathway, binding of U1snRNP (Stark et al. 2001) to the 5' splice site (5'ss) (Mount et al. 1983; Zhang 1998; Ast 2004) represents one of the key events in triggering this process (Krainer 1997; Burge et al. 1999). Although several studies have investigated the basic properties of this event (Zhuang and Weiner 1986; Seraphin et al. 1988; Siliciano and Guthrie 1988; Seraphin and Rosbash 1989; Michaud and Reed 1991; Horowitz and Krainer 1994; Tarn and Steitz 1994), much still remains to be clarified regarding its many peculiarities during constitutive and alternative splicing (Eperon et al. 2000; Du and Rosbash 2001, 2002; Liu 2002; Lund and Kjems 2002; Freund et al. 2003, 2005; Roca et al. 2005).

Notwithstanding this complexity, mutations that destroy natural donor sequences are generally observed to cause the skipping of their associated exon from the splicing queue

(Krawczak et al. 1992). However, since the earliest descriptions of splicing defects (Treisman et al. 1983; Wieringa et al. 1983), it has also been observed that in a rather high proportion of cases the inactivation of natural donor splice sites can lead to the activation of cryptic splice site sequences (for an up-to-date list, see Roca et al. 2003). This observation is in keeping with recent analyses that show that potential splice site sequences are particularly abundant in pre-mRNA molecules (Sun and Chasin 2000) and it is, therefore, to be expected that some of these sequences may be used following the inactivation of the rightful donor site. Indeed, several studies have suggested that cryptic splice site scores are generally lower than those calculated for the natural splice sites they replace (Lear et al. 1990; Ars et al. 2000). However, this is not always the case (Treisman et al. 1983) and recent evidence has pointed out that intrinsic features and local context of these cryptic sites allow the splicing machinery to actively discriminate against their usage and in favor of natural sites (Roca et al. 2003). In this respect, it has to be noted that natural and cryptic sites share many factors that have been known to act on the general splicing pathway and have been described to affect cryptic splicing events depending on

Reprint requests to: Emanuele Buratti, Padriciano 99, 34012 Trieste, Italy; e-mail: buratti@icgeb.org; fax: +39-040-3757361.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2269306>.

local context. For example, RNA secondary structure (Buratti and Baralle 2004) has been proposed to act as specific modifiers of cryptic splice site usage in adenovirus (Domenjoud et al. 1991, 1993). In addition, SR protein levels, and in particular SF2/ASF, represent a very powerful general modifier of splicing events (Graveley 2000; Bourgeois et al. 2004) and have been demonstrated to affect relative cryptic splice site usage in the human β -globin gene both in vitro and in vivo (Krainer et al. 1990; Caceres et al. 1994). Recently, research on cryptic splice sites usage has also provided additional insight regarding the evolutionary mechanisms of intron gain/loss (Stoltzfus 2004), by suggesting that in the *actin* genes (Sadusky et al. 2004) cryptic splice sites act as preferential sites for intron insertion. Therefore, a better understanding of the splicing mechanisms that favor one cryptic splice site sequence over other competing cryptic sites may also provide additional functional explanations for this kind of insertional events.

Fibrinogen is a homodimeric glycoprotein composed of pairs of A α -, B β -, and γ -polypeptide chains that are encoded by the single copy *FGA*, *FGB*, and *FGG* genes, respectively, clustered on 4q31.3–32.1 chromosomal region (UCSC Genome Browser [<http://genome.ucsc.edu/>]). So far, nine splicing mutations spread over the three fibrinogen genes account for 24% of gene alterations underlying congenital afibrinogenemia, a rare inherited coagulation disorder characterized by lack of plasma fibrinogen (Mannucci et al. 2004). The phenotypic effects on mRNA processing have been evaluated for some of these mutations and resulted either in the creation of de novo 5' and 3' splice sites (Spena et al. 2002; Asselta et al. 2004) or in the inactivation of authentic 5'ss (Asselta et al. 2000; Margaglione et al. 2000; Attanasio et al. 2001; Spena et al. 2002). For these latter genetic defects, no correlation between the localization of point mutations within the 5'ss consensus sequences and the splice outcomes (cryptic site activation, exon skipping, and intron inclusion) has been so far established.

In this work, we describe the first identification of a SF2/ASF binding sequence within *FGB* exon 7, which is not needed for normal constitutive splicing but can affect specific cryptic site usage following the IVS7 + 1G>T mutation in its natural 5'ss sequence (Spena et al. 2002).

RESULTS AND DISCUSSION

Analyzing the sequence features enabling activation of cryptic splice sites in *FGB* exon 7

In a previous work we characterized the mRNA aberrant pattern generated by the IVS7 + 1G>T mutation located in the first nucleotide of intron 7 of the fibrinogen B β -chain gene (*FGB*), which was first identified as causative alter-

ation in a patient affected by congenital afibrinogenemia (Spena et al. 2002). Minigene analysis revealed that beside exon 7 skipping this mutation caused the activation of three cryptic donor splice sites, localized in the upstream exon at 106 nt (c1), 40 nt (c2), and 24 nt (c3) from the physiologic 5'ss (Fig. 1A). Interestingly, cryptic splice site activation represents the main consequence of this mutation as opposed to exon skipping, accounting for 72% of the total aberrant splice products (Spena et al. 2002). In this work, we have investigated the molecular mechanisms that lead to the selection of these specific cryptic 5'ss.

In order to evaluate the in silico relevance of these cryptic splice site sequences, all potential 5'ss located in the upstream exon 7 and in the downstream intron 7 of the authentic site were predicted using the Neural Network (NN) (Reese et al. 1997) and MaxEntScan (Yeo and Burge 2004) tools. As shown in Figure 1A, only one (c1) of the three exonic cryptic sites activated in vivo was recognized by the NN program. This tool also suggested two additional potential intronic donor sites, located 158 nt (p1) and 549 nt (p2) from the authentic site, that are never used by the splicing machinery. The strength of all cryptic and predicted sites was also compared by three other methods—the maximum entropy model (MAXENT), the maximum dependence decomposition model (MDD), and the first-order Markov model (MM)—that require the prior knowledge of the input sequence to be tested. Also in this case, intrinsic sequence differences were found to be generally consistent with the relative cryptic 5'ss activation observed when the IVS7 + 1G>T mutation affects the authentic site (Spena et al. 2002). In fact, the MAXENT, MDD, and MM scores were consistently higher for the c1 site with respect to the c2 and c3 splice sites (Fig. 1A), reflecting the fact that in vivo this cryptic site is used 57.3% of the time, while c2 and c3 only 14.6% and 2.2% of the time, respectively (Spena et al. 2002). Nevertheless, also these methods fail to reveal any difference between the cryptic c1 splice site and the potential p1 site, which is never used in vivo (Fig. 1A). In conclusion, although these combined prediction methods identify correctly the major cryptic splice site (c1) they fail to adequately describe the overall pattern of cryptic splice site activation in *FGB* exon 7.

This is consistent with the observation that in some cases local context may play a decisive role in cryptic splice site activation (Roca et al. 2003). In these cases, it has thus been proposed that other factors beside splice site strength, such as the presence of ESE sequences, may act as a potential modifier of splice site selection (Cartegni et al. 2002; Wang et al. 2005). For this reason it was thus decided to determine the putative presence of such sequences within the exon 7 itself.

To this aim, a preliminary computer-assisted analysis was performed by the ESE finder program (Cartegni et al.

FIGURE 1. (A) (Upper panel) Schematic diagram of the human *FGB* minigene spanning from exon 7 to exon 8 (white and black boxes, respectively) indicating the position (drawn to scale) and sequence of the three cryptic splice sites (c1, c2, and c3) used when the natural donor splice site is abolished following the IVS7 + 1G>T mutation (black circle) and of the two potential donor sites (p1 and p2) identified by in silico analysis but never used in vivo. (Lower panel) Individual scores for each of these cryptic and potential splice sites obtained by a panel of donor site prediction programs (NN, MAXENT, MDD, and MM); np denotes not predicted sites. (B) (Upper panel) Outline of the wild-type *FGB* minigene used in transfection experiments. Rectangles, drawn to scale, indicate exons (gray, exon 6; white, exon 7; and black, exon 8); single lines, introns. Dotted lines indicate splicing products; arrows, the primer couple used to amplify the splicing products by RT-PCR. (Middle panel) *FGB* genomic region (numbered according to GenBank [accession no. M64983]) containing exon 7 (in uppercase letters) with the mutations (numbered above the sequence) introduced in its wild-type sequence to remove all potential SR binding sites identified by the ESEfinder program. In mutant 1 potential binding sites were disrupted by point mutations (reported below the sequence); in mutants 2–8, by deletion (the deleted sequence is underlined in each case). Sequences of the three cryptic sites (c1, c2, and c3) are bold and shaded in gray. (Lower panel) Effect of all these mutations (lanes 1–8) with respect to the wild-type minigene (wt) following transfection of each minigene in HeLa cells. RT-PCR products were run on a 2%-agarose gel electrophoresis. M indicates molecular weight marker (pUC8HaeIII).

2003), which enabled the identification of several overlapping high-score ESEs motifs, potentially recognizable by four SR proteins (SF2/ASF, SC35, SRp40, and SRp55), spread over the entire exon 7 sequence (data not shown). In order to evaluate the functional relevance of these elements in their natural context, the mammalian expression vector

pT-B β -wt (see Fig. 1B, upper panel, for a schematic diagram of the wild-type *FGB* minigene) was used as a template to generate eight recombinant mutant constructs lacking groups of predicted ESEs elements that were inactivated either by single-nucleotide mutagenesis (mutant 1) or by deletion (mutants 2–8) (Fig. 1B, middle panel). The wild-type

and mutant vectors were independently transfected in HeLa cells (not expressing fibrinogen), and total RNAs were extracted and analyzed by RT-PCR. After agarose gel electrophoresis it was observed that all RT-PCR products showed complete exon inclusion (Fig. 1B, lower panel), and the slight reduction in length of fragments corresponding to mutants 2–8 was consistent with the loss in exon 7 of the deleted regions. These results suggested that in the wild-type context there are no predictable ESE motifs that affect natural exon 7 inclusion.

Recruitment of SR proteins by *FGB* exon 7

In parallel, however, the potential SR binding ability of exon 7 was also evaluated by immunoprecipitation experiments. For this purpose, the entire exon 7 and three overlapping regions of the same exon (namely fragments A, B, and C) (Fig. 2A, upper panel) were cloned into the expression vector pBluescript II KS. After linearization and in vitro transcription, each RNA was analyzed by UV cross-linking with total nuclear extract from HeLa cells (Fig. 2A, lower panel 1). Three batches of these UV-cross-linked samples were immunoprecipitated with monoclonal antibodies against SF2/ASF (mAb 96), the phosphorylated RS domain (mAb 1H4), and SC35 (mAb anti-SC35) (Fig. 2A, lower panels 2,3,4). The results showed that SF2/ASF and SRp40 proteins are specifically immunoprecipitated by exon 7, while very weak signals were detected for the SRp55, SRp75, and SC35 proteins (Fig. 2A, lower panels 2–4).

Because the interpretation of this assay may be complicated by possible sequence-specific cross-linking bias, we also tested the interaction of SR proteins with *FGB* exon 7 using a pull-down protocol that has already been previously used to investigate a number of RNA–protein interactions in the *CFTR* and *NF-1* genes (Buratti et al. 2001, 2004a). In this assay, the RNA was covalently coupled to adipic acid dehydrazide beads and incubated with nuclear extract to pull down RNA-bound factors. The RNA-bound factors were then run on a SDS-PAGE gel, Western blotted, and recognized by the same SR specific antibodies used in the immunoprecipitation analyses. As shown in Figure 2B, the results demonstrated that the exon 7 sequence is capable of pulling down SF2/ASF very efficiently (panel 1) compared with the input amount of nuclear extract (1/5) used in this assay. Conversely, the efficiency of pull-down was much lower for SRp40 (panel 2) and no pull-down signal was observed for SRp55, SRp75 (panel 2), and SC35 (panel 3). It should be noted that SR protein signal assignment for the 1H4 antibody (panel 2) has been achieved by comparing the pattern in the nuclear extract lane with previously published data (Neugebauer et al. 1995).

In particular, as SF2/ASF immunoprecipitation was enriched in fragment C (Fig. 2A, lower panel 2) in order to better localize the binding sites for SF2/ASF, the fragment C was divided into three overlapping regions (fragments C1,

C2, and C3) (Fig. 2C, left panel) and analyzed by total UV cross-linking with HeLa nuclear extract and immunoprecipitation (Fig. 2C, right panels). The band corresponding to the labeled SF2/ASF protein displayed a preferential binding in both fragments C1 and C3, allowing to map the ESE binding site for SF2/ASF in the 36-nt overlapping region (encompassing nucleotides 7074–7109, according to GenBank accession no. M64983) (Fig. 2C). Interestingly, although this sequence did not display any particularly strong SR consensus binding sequence (as determined using ESEfinder), it was highly enriched in short hexameric sequences that represent candidate exonic splicing enhancers according to the RESCUE-ESE program (Fairbrother et al. 2002, 2004; data not shown). It is tempting to speculate that a connection may exist between binding of this protein and this particular set of RESCUE-ESE motifs, although a recent analysis of SF2/ASF ESEfinder motifs with RESCUE-ESE motifs showed that the output of these prediction programs did not overlap beyond what was to be expected by chance (Wang et al. 2005).

Deletion of the SF2/ASF binding region modifies cryptic splice site selection in the IVS7 + 1G>T mutant

To functionally examine the role of SF2/ASF on cryptic site selection, the two B β -minigene constructs containing either the natural or the mutant donor site (pT-B β -wt and pT-B β -IVS7 + 1G>T) were used as templates to generate two mutants lacking the identified 36-bp binding region for SF2/ASF (pT-B β - Δ wt and pT-B β - Δ IVS7 + 1G>T). These four vectors were transiently transfected in HeLa cells and the mRNAs reversely transcribed and PCR-amplified using the highly sensitive fluorescent hot-stop technique. The resulting mRNA products were then separated by agarose gel electrophoresis (Fig. 3A). In the case of the Δ wt minigene a unique band, slightly lower than the expected wild-type one (454 bp), was visualized (Fig. 3A, lane 2). On the other hand, concerning the Δ IVS7 + 1G>T minigene four aberrant products were generated (Fig. 3A, lane 4) that could reflect the cryptic splice sites activation and the exon skipping previously characterized in the IVS7 + 1G>T minigene (Spena et al. 2002). Of these products, the shorter one (es band) had the same molecular weight as the exon skipping product, while the other three (Δ c1, Δ c2, and Δ c3 products) showed a shifted pattern with respect to the c1, c2, and c3 bands observed in the IVS7 + 1G>T minigene (Fig. 3A, lane 3). A further characterization of these splicing events was performed by subcloning and sequencing all Δ wt and Δ IVS7 + 1G>T products (data not shown). This qualitative analysis confirmed a correct splicing for the Δ wt product and, in the case of Δ IVS7 + 1G>T minigene, a pattern of cryptic site activation and exon skipping identical to the one observed in the IVS7 + 1G>T sample. What seemed to differ, however, was the relative abundance

of the products originating from cryptic splice site usage (for example, compare the intensity of the $\Delta c1$ product in lane 4 with the corresponding fragment c1 in lane 3).

Therefore, to obtain sizing and semiquantitation of mRNA species, the fluorochrome-labeled products were

separated by an automated DNA sequencer (Fig. 3B, upper panel). First of all it should be noted that, as expected, Δwt as well as $\Delta c1$, $\Delta c2$, and $\Delta c3$ products were 36 bp shorter than the corresponding wt, c1, c2, and c3 fragments (418, 394, 378, and 312 bp vs. 454, 430, 414, and 348 bp,

respectively), while the smaller product had the same length (168 bp) of the es fragment (Fig. 3B, lower panel).

Most importantly, however, this analysis allowed the semiquantitation of both IVS7 + 1G>T and Δ IVS7 + 1G>T mutant splicing products that was performed by measuring for each samples fluorescent-peak areas (Fig. 3B). The results, obtained from three independent transfection experiments, confirmed that the 36-bp deletion, although not affecting total ratio of cryptic site activation (68.5% vs. 76.2%), alters their relative use. In particular, we observed a great reduction in the distal (c1) cryptic ss activation (15.0% vs. 43.6%) beside a small increase in the other two (c2 and c3) 5'ss usage (30.0% vs. 17.1% and 23.5% vs. 15.5%, respectively) and in the exon skipping event (31.4% vs. 23.8%) (Fig. 3B, lower panel).

In order to further characterize the functional specificity of this sequence, we also tested the effect of mutations 1, 2, 3, and 4 (Fig. 1B, middle panel) on cryptic site usage in the presence of the IVS7 + 1G>T mutation. As shown in Figure 3C, none of these alterations (point mutations and deletions) is capable of affecting significantly cryptic site usage with respect to the IVS7 + 1G>T minigene. This result confirmed that the observed functional effects of the Δ deletion are specific for this sequence and are not simply caused by other effects such as the reduction in exon length or the simple bringing closer of some other negative RNA splicing regulator.

In parallel, we also wished to test functionally the importance of SR binding and in particular of SF2/ASF binding in this particular position on *FGB* exon 7. To this end, the Δ sequence was replaced with a well-characterized ESE sequence (EDA hTot) from the fibronectin EDA exon that has been previously described to be a good binder of SR proteins and with the same sequence containing a GAAGAAGA deletion (EDA Δ 2e) that was also previously shown to abolish ESE activity in the fibronectin context (Caputi et al. 1994; Buratti et al. 2004b; Fig. 3D, left panel). As shown in Figure 3D, right panel, transfection of the minigene Δ IVS7 + 1G>T + EDA hTot efficiently rescued usage of the c1 cryptic splice site from the 16.0% observed with the Δ IVS7 + 1G>T minigene to 63.4%, a value that also represents a substantial increase over its use in the

undeleted IVS7 + 1G>T minigene (39.4%). Furthermore, in keeping with the proposed role played by SF2/ASF binding in this position, the minigene Δ IVS7 + 1G>T + EDA Δ 2e carrying the GAAGAAGA deletion showed a reduction of c1 usage with respect to the minigene carrying the EDA ESE wild-type sequence. The reason why the decrease in c1 usage is not as great as might be expected (50.0%) probably resides in the fact that, in the highly compromised *FGB* exon 7 context, the EDA Δ 2e sequence maintains a certain degree of enhancer activity. Taken together, these data functionally demonstrate that positioning a heterologous SR protein binding sequence between nucleotides 7074 and 7109 can successfully influence cryptic splice site usage.

In conclusion, these results suggest that the mRNA aberrant pattern generated by the mutant IVS7 + 1G>T substrate relies for its relative usage on binding of the SF2/ASF protein to a specific motif located in *FGB* exon 7 upstream of the exonic cryptic splice sites (Fig. 3E). The observation that SF2/ASF can alter the relative cryptic site usage (but not their identity) is in agreement with previous data obtained for the β -globin gene (Krainer et al. 1990; Caceres et al. 1994). In our case, however, the functional effect of this protein on the c1 cryptic splice site is mediated by its strong binding site within exon 7, rather than on the general cellular levels of SF2/ASF. As this binding site, the first identified in *FGB*, does not seem to participate actively in the exon 7 wild-type context, this result suggests the existence of splicing regulatory sequences that can only reveal themselves when normal splice site determination is inactivated. In this respect, our work provides experimental evidence concerning the identity of one important local context factor (i.e., cryptic ESE sequences) that does not normally affect wild-type splicing processes but that can nonetheless heavily influence cryptic splice site usage when conditions allow. In this respect, supporting evidence regarding the potential involvement of cryptic ESEs in donor site activation has also been recently published in connection with a disease-causing mutation observed to occur in the E1 α PDH pre-mRNA. In this mRNA, activation of a cryptic donor site upstream of exon 7 has been demonstrated to occur following the introduction of a point

FIGURE 2. (A) (Upper panel) Schematic diagram of *FGB* exon 7 with the three sequences (A, B, and C) into which it has been divided. Borders of fragments, drawn to scale, are indicated and numbered according to GenBank (accession no. M64983). (Lower panel) UV cross-linking profiles (panel 1) of the RNAs obtained from the four sequences (7, A, B, and C). The electrophoretic mobility of prestained marker (Broad Range, New England Biolabs) is shown on the left. Immunoprecipitation profiles of each UV-cross-linked sample shown at left, with specific monoclonal antibodies against different SR proteins: SF2/ASF (mAb 96, panel 2), the phosphorylated RS domain (mAb 1H4, panel 3), and SC35 (anti-SC35, panel 4). The mobility of the SR proteins is indicated on the left. (B) Western blot panels of SR proteins bounded to exon 7 resulting to pull-down analysis using coated and uncoated (as control) adipic acid beads. SR proteins were detected by their respective monoclonal antibodies against SF2/ASF (mAb 96, panel 1), the phosphorylated RS domain (mAb 1H4, panel 2), and SC35 (anti-SC35, panel 3). Input represents 20% of the total nuclear extract load during each pull-down experiment. (C) (Left panel) Schematic representation of the three sequences (C1, C2, and C3) used to map the SF2/ASF binding site (hatched) on fragment C of exon 7. Ends of fragments, drawn to scale, are indicated and numbered according to GenBank (accession no. M64983). Sequence of the 36-bp binding region is reported. (Middle panel) Total UV cross-linking analysis of these three RNAs. (Right panel) Immunoprecipitation analysis of these samples with mAb 96 (specific for SF2/ASF).

mutation that strengthens a weak SC35 binding site that was previously present on this pre-mRNA (Gabut et al. 2005).

Finally, our observation that introduction of a heterologous ESE can successfully alter cryptic splice site usage also

possesses considerable relevance with regards to potential methods for the control of aberrant splicing. In fact, even without changing the context of the mutation that causes the disease, it may be advantageous to favor activation of one cryptic splice site over others in order to avoid, for

FIGURE 3. (Legend on next page)

example, the introduction of premature stop codons. In this respect, it is important to note that the introduction of bifunctional oligonucleotides to provide *trans*-acting enhancer ability has already been proved successful in the recovery of *SMN2* expression both in vivo and in vitro (Cartegni and Krainer 2003; Skordis et al. 2003) and in the modulation of *bcl-x* alternatively spliced transcripts (Wilusz et al. 2005).

MATERIALS AND METHODS

Computer prediction analysis

Searching for potential donor splice sites in the *FGB* region spanning from exon 7 to intron 7 was accomplished using the NN splice site prediction tool (http://www.fruitfly.org/seq_tools/splice.html). Scores of the 9-nt sequences, corresponding either to the NN predicted and the cryptic 5'ss, were calculated by means of the MaxEntScan program (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) by selecting for the analyses the MAXENT, the MDD, or the MM. Finally, the presence of potential SF2/ASF, SC35, SRp40, and SRp55 binding sites was predicted by the ESEfinder program (<http://rulai.cshl.edu/tools/ESE/>). All programs are freely available at the indicated Web sites.

Minigene construction

The recombinant constructs pT-B β -wt and pT-B β -IVS7 + 1G>T, containing the wild-type and the mutant IVS + 1G>T human *FGB* genomic region spanning from exon 6 to exon 8, have been previously described (Spena et al. 2002). Mutations 1–8, Δ , and EDA hTot- Δ 2e were inserted in the *FGB* exon 7 of the pT-B β -wt and/or pT-B β -IVS7 + 1G>T constructs by site-directed mutagenesis with oligonucleotides (available upon request) carrying point mutations (for mutant 1), 22–36 bp deletion (for mutants 2–8 and Δ), or 23–31 bp insertion (for mutants EDA hTot- Δ 2e), as indicated in Figures 1B, 2C, and 3D. Each mutagenesis reaction was carried out in 18 PCR cycles by using the Pfu Turbo

(Stratagene) and following the manufacturer's instructions. Sequences of exon 7 and fragments A, B, C, C1, C2, and C3, whose lengths are indicated in Figure 2, were amplified from the minigene construct pT-B β -wt by using sense and antisense primers (available upon request) carrying the SacI and BamHI restriction sites at the 5' end, respectively, and were cloned into the linearized SacI/BamHI pBluescript II KS plasmid. All constructs were purified by the Qiaprep Maxiprep Kit (Qiagen) and checked by direct sequencing.

Cell culture, transfection, and RNA extraction

Human cervix carcinoma HeLa cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics (100 IU/mL penicillin and 100 μ g/mL streptomycin). Cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C and cultured according to standard procedures. Transient transfections were performed in semiconfluent cells (2×10^6 cells in 10-cm dishes) by means of the calcium phosphate method, essentially as described (Wigler et al. 1978). Twenty micrograms (20 μ g) of each specific construct was independently transfected, and, 16 h after transfection, cells were washed twice with phosphate-buffered saline and harvested in fresh medium for an additional 48 h before RNA extraction. Total RNA was isolated by a single-step extraction method by using the RNeasy reagent (Ambion). Three independent transfection experiments were carried out for each construct.

RT-PCR and fluorescent hot-stop PCR analyses

cDNA was synthesized from 1 μ g total RNA of each samples by means of random nonamers (Sigma-Aldrich) and the ImProm-II Reverse Transcriptase (Promega), according to the manufacturer's instructions. To analyze splice products, an aliquot (2.5 μ L) of the total reaction volume (20 μ L) was used as template in a standard PCR amplification with the *FGB* exonic primer couple FGB-Ex6-F (5'-agtgtattcagaaccgtcaag-3') and FGB-Ex8-R (5'-tcaccaccgtcttcttag-3'). To allow semiquantitative analysis of aberrant splicing products, a fluorescent-hot stop PCR (Asselta et al. 2000) was accomplished by adding to each reaction mixture 0.4 μ M FGB-Ex8-R 6-Fam-labeled primer at the final PCR cycle. Total number

FIGURE 3. (A) Agarose (2%) gel electrophoresis of RT-PCR products amplified from total RNA extracted from HeLa cells after transfection either with the wild-type *FGB* minigene (wt, lane 1), the wild-type minigene carrying the deletion of the SF2/ASF binding region (Δ wt, lane 2), the minigene carrying the +1G>T substitution in the natural donor splice site (IVS7 + 1G>T, lane 3), and the minigene carrying this mutation together with the deletion of the SF2/ASF binding region (Δ IVS7 + 1G>T, lane 4). M indicates molecular weight marker (pUC8HaeIII). (B) (Upper panel) GeneScan Analysis windows of the capillary-electrophoretic runs of the same fluorochrome labeled RT-PCR products showed in A. Molecular weight standard peaks are in gray, while peaks corresponding to the labeled fragments, indicated by arrows, are in black. Lengths of molecular weight fragments (bp) are reported on the left. (Lower panel) Size (bp) and relative amounts (%) of the fluorochrome labeled RT-PCR products indicated in the upper panel, evaluated by means of the GeneScan software. The relative amounts represent the average of three independent experiments with standard deviation values. Levels of c1 and Δ c1 products are shaded in gray. (C) Functional specificity of SF2/ASF binding region tested by evaluating the effect of mutations 1–4 (see Fig. 1B, middle panel) on cryptic splice site usage in the IVS7 + 1G>T minigene with respect to the same minigene following transfection of each construct in HeLa cells. RT-PCR products were run on a 2%-agarose gel electrophoresis. M indicates molecular weight marker (pUC8HaeIII). (D) (Left panel) Schematic representation of the IVS7 + 1G>T minigene used to replace the deleted (Δ) binding region of SF2/ASF by the heterologous fibronectin EDA ESE sequence (EDA hTot) and the deleted EDA ESE sequence (EDA Δ 2e) used as a control. Black circle indicates the IVS7 + 1G>T mutation; arrows, the primer couple used to amplify the splicing products by RT-PCR. (Right panel) Agarose (2%) gel electrophoresis of RT-PCR products amplified from total RNA extracted from HeLa cells expressing the heterologous ESE-carrying minigene (Δ IVS7 + 1G>T + EDA hTot) as compared with the effects of the same minigene carrying the GAAGAAGA deletion (Δ IVS7 + 1G>T + EDA Δ 2e) and the IVS7 + 1G>T and Δ IVS7 + 1G>T minigenes. The relative amounts (%) of c1 usage for each minigene are indicated on the bottom. M indicates molecular weight marker (pUC8HaeIII). (E) Schematic diagram of the switch in relative cryptic splice site usage (depicted by thicker dotted lines) following the deletion (Δ) of the SF2/ASF binding sites in the IVS7 + 1G>T and Δ IVS7 + 1G>T constructs.

of cycles was also minimized to maintain linearity. Labeled amplicons were separated on an ABI-3100 Genetic Analyzer sequencer (Applied Biosystems) and analyzed by the GenScan Analysis software 3.1 (Applied Biosystems).

Subcloning and sequencing

An aliquot (5 μ L) of RT-PCR products was drawn prior to the addition of the labeled primer and subcloned into the pGEM-T Easy Vector (Promega). Screening of recombinant clones was performed by PCR, as described (Spena et al. 2002), and products were directly sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Sequencing products were analyzed on an ABI-3100 Genetic Analyzer (Applied Biosystems), and sequence analyses were accomplished by means of Factura and Sequence Navigator packages (Applied Biosystems).

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

To generate RNA probe of exon 7 and fragments A, B, C, C1, C2, and C3, the corresponding pBluescript II KS recombinant constructs were linearized at the SmaI site and transcribed with T7 RNA Polymerase (Pharmacia Biotech) in the presence of α^{32} P-UTP, according to standard procedures. The UV cross-linking assay was performed by incubating $5 \times 10^5 - 1 \times 10^6$ cpm labeled-RNA probes with 225 μ g of HeLa nuclear extracts (4C, Biotech) and 100 μ g heparin in a 20- μ L final reaction volume containing 20 mM Hepes (pH 7.9), 72 mM KCl, 1.5 mM MgCl₂, 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 800,000 kJ UV light for 5 min by using a BIO-LINK (Euroclone). Unbound RNA was digested with 30 μ g of RNase A (Sigma) for 30 min at 37°C and loaded onto a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) gel or, for the immunoprecipitation analysis, incubated for 2 h at 4°C on a rotator wheel with 150 μ L of IP buffer (20 mM Tris at pH 8.0, 300 mM NaCl, 1 mM EDTA, 0.25% NP-40) and 1 μ L of monoclonal antibodies anti-SF2/ASF (mAb 96) (Zymed Laboratories Inc), anti-RS phosphorylated domain (mAb 1H4) (Zymed Laboratories Inc), or anti-SC35 mAb (Sigma). Each mixture was then incubated with 30 μ L of Protein A/G-Plus Agarose (Santa Cruz Biotechnologies) at 4°C overnight. Beads were collected by centrifugation, washed four times with 1.5 mL of IP buffer, and then loaded onto a SDS-11% PAGE gel. Gels were run at a constant 30 mA for $\sim$ 3.5 h, dried under vacuum, and exposed for 4 d with a BioMax Screen (Kodak).

Pull-down and Western blot protocol

In order to identify which SR proteins bind exon 7, a pull-down assay using adipic acid dehydrazide beads was used. Briefly, 500 pmol of the target RNA ($\sim$ 5 μ g of a 100-mer RNA) were placed in a 400- μ L reaction mixture containing 100 mM NaOAC (pH 5.2) and 5 mM sodium m-periodate (Sigma), incubated for 1 h in the dark at room temperature, ethanol-precipitated, and resuspended in 100 μ L of 0.1 M NaOAC (pH 5.2). To this RNA 300 μ L of adipic acid dehydrazide agarose bead 50% slurry (Sigma) equil-

ibrated in 100 mM NaOAC (pH 5.2) was added, and the mix was incubated for 12 h at 4°C on a rotator. The beads with the bound RNA were then pelleted, washed two times with 1 mL of 2 M NaCl, and equilibrated in washing buffer (5 mM HEPES at pH 7.9, 1 mM MgCl₂, 0.8 mM Magnesium acetate). They were then incubated on a rotator with a protein mixture containing $\sim$ 1 mg of HeLa cell nuclear extract (4C, Biotech) for 30 min at room temperature in 0.6 mL final volume. The resulting 1 $\times$ reaction buffer was 20 mM HEPES-KOH (pH 7.0), 6.5% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, heparin was also added to a final concentration of 0.75 μ g/ μ L before addition of the nuclear extract to the beads. The beads were then pelleted by centrifugation at 3000 rpm for 3 min in an Eppendorf minifuge and washed four times with 1.5 mL of washing buffer before addition of SDS sample buffer and loading on a SDS-10% PAGE gel. Western blots were then performed according to standard protocols on a Hybond-C Extra membrane (Amersham Biosciences) and blocked by addition of Western Blot Blocking Reagent (Roche) according to manufacturer's instructions. Antibody recognition was then performed using the mouse monoclonal antibodies described in the immunoprecipitation protocol at a dilution of 1:1000. Western blots signals were then detected with an enhanced chemiluminescence kit (ECLplus, Amersham Pharmacia Biotech).

ACKNOWLEDGMENTS

This work was supported by the Telethon Onlus Foundation (Italy) (grant nos. GGP02453 and GGP030261) and by FIRB (RBNE01W9PM). S.S. is a recipient of a Telethon fellowship. We thank Prof. F.E. Baralle for helpful advice and critical reading of the manuscript.

Received October 21, 2005; accepted February 27, 2006.

REFERENCES

- Ars, E., Serra, E., Garcia, J., Kruyer, H., Gaona, A., Lazaro, C., and Estivill, X. 2000. Mutations affecting mRNA splicing are the most common molecular defects in patients with neurofibromatosis type 1. *Hum. Mol. Genet.* **9**: 237–247.
- Asselta, R., Duga, S., Simonc, T., Malcovati, M., Santagostino, E., Giangrande, P.L., Mannucci, P.M., and Tenchini, M.L. 2000. Afibrinogenemia: First identification of a splicing mutation in the fibrinogen gamma chain gene leading to a major gamma chain truncation. *Blood* **96**: 2496–2500.
- Asselta, R., Duga, S., Spena, S., Peyvandi, F., Castaman, G., Malcovati, M., Mannucci, P.M., and Tenchini, M.L. 2004. Missense or splicing mutation? The case of a fibrinogen B β -chain mutation causing severe hypofibrinogenemia. *Blood* **103**: 3051–3054.
- Ast, G. 2004. How did alternative splicing evolve? *Nat. Rev. Genet.* **5**: 773–782.
- Attanasio, C., de Moerloose, P., Antonarakis, S.E., Morris, M.A., and Neerman-Arbez, M. 2001. Activation of multiple cryptic donor splice sites by the common congenital afibrinogenemia mutation, FGA IVS4 + 1 G $\rightarrow$ T. *Blood* **97**: 1879–1881.
- Bourgeois, C.F., Lejeune, F., and Stevenin, J. 2004. Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **78**: 37–88.
- Buratti, E. and Baralle, F.E. 2004. Influence of RNA secondary structure on the pre-mRNA splicing process. *Mol. Cell. Biol.* **24**: 10505–10514.

- Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M., and Baralle, F.E. 2001. Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO J.* **20**: 1774–1784.
- Buratti, E., Baralle, M., De Conti, L., Baralle, D., Romano, M., Ayala, Y.M., and Baralle, F.E. 2004a. hnRNP H binding at the 5' splice site correlates with the pathological effect of two intronic mutations in the *NF-1* and *TSHB* genes. *Nucleic Acids Res.* **32**: 4224–4236.
- Buratti, E., Muro, A.F., Giombi, M., Gherbassi, D., Iaconcig, A., and Baralle, F.E. 2004b. RNA folding affects the recruitment of SR proteins by mouse and human polypurinic enhancer elements in the fibronectin EDA exon. *Mol. Cell. Biol.* **24**: 1387–1400.
- Burge, C.B., Tuschl, T., and Sharp, P.A. 1999. *Splicing of precursors to mRNAs by the spliceosome*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Caceres, J.F., Stamm, S., Helfman, D.M., and Krainer, A.R. 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. *Science* **265**: 1706–1709.
- Caputi, M., Casari, G., Guenzi, S., Tagliabue, R., Sidoli, A., Melo, C.A., and Baralle, F.E. 1994. A novel bipartite splicing enhancer modulates the differential processing of the human fibronectin EDA exon. *Nucleic Acids Res.* **22**: 1018–1022.
- Cartegni, L. and Krainer, A.R. 2003. Correction of disease-associated exon skipping by synthetic exon-specific activators. *Nat. Struct. Biol.* **10**: 120–125.
- Cartegni, L., Chew, S.L., and Krainer, A.R. 2002. Listening to silence and understanding nonsense: Exonic mutations that affect splicing. *Nat. Rev. Genet.* **3**: 285–298.
- Cartegni, L., Wang, J., Zhu, Z., Zhang, M.Q., and Krainer, A.R. 2003. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* **31**: 3568–3571.
- Domenjoud, L., Gallinaro, H., Kister, L., Meyer, S., and Jacob, M. 1991. Identification of a specific exon sequence that is a major determinant in the selection between a natural and a cryptic 5' splice site. *Mol. Cell. Biol.* **11**: 4581–4590.
- Domenjoud, L., Kister, L., Gallinaro, H., and Jacob, M. 1993. Selection between a natural and a cryptic 5' splice site: A kinetic study of the effect of upstream exon sequences. *Gene Expr.* **3**: 83–94.
- Du, H. and Rosbash, M. 2001. Yeast U1 snRNP-pre-mRNA complex formation without U1snRNA-pre-mRNA base pairing. *RNA* **7**: 133–142.
- . 2002. The U1 snRNP protein U1C recognizes the 5' splice site in the absence of base pairing. *Nature* **419**: 86–90.
- Eperon, I.C., Makarova, O.V., Mayeda, A., Munroe, S.H., Caceres, J.F., Hayward, D.G., and Krainer, A.R. 2000. Selection of alternative 5' splice sites: Role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1. *Mol. Cell. Biol.* **20**: 8303–8318.
- Fairbrother, W.G., Yeh, R.F., Sharp, P.A., and Burge, C.B. 2002. Predictive identification of exonic splicing enhancers in human genes. *Science* **297**: 1007–1013.
- Fairbrother, W.G., Yeo, G.W., Yeh, R., Goldstein, P., Mawson, M., Sharp, P.A., and Burge, C.B. 2004. RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res.* **32**: W187–W190.
- Freund, M., Asang, C., Kammler, S., Konermann, C., Krummheuer, J., Hipp, M., Meyer, I., Gierling, W., Theiss, S., Preuss, T., et al. 2003. A novel approach to describe a U1 snRNA binding site. *Nucleic Acids Res.* **31**: 6963–6975.
- Freund, M., Hicks, M.J., Konermann, C., Otte, M., Hertel, K.J., and Schaal, H. 2005. Extended base pair complementarity between U1 snRNA and the 5' splice site does not inhibit splicing in higher eukaryotes, but rather increases 5' splice site recognition. *Nucleic Acids Res.* **33**: 5112–5119.
- Gabut, M., Mine, M., Marsac, C., Brivet, M., Tazi, J., and Soret, J. 2005. The SR protein SC35 is responsible for aberrant splicing of the *El α* pyruvate dehydrogenase mRNA in a case of mental retardation with lactic acidosis. *Mol. Cell. Biol.* **25**: 3286–3294.
- Graveley, B.R. 2000. Sorting out the complexity of SR protein functions. *RNA* **6**: 1197–1211.
- Horowitz, D.S. and Krainer, A.R. 1994. Mechanisms for selecting 5' splice sites in mammalian pre-mRNA splicing. *Trends Genet.* **10**: 100–106.
- Krainer, A.R. 1997. *Eukaryotic mRNA processing*. Oxford University Press Inc., New York.
- Krainer, A.R., Conway, G.C., and Kozak, D. 1990. The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. *Cell* **62**: 35–42.
- Krawczak, M., Reiss, J., and Cooper, D.N. 1992. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: Causes and consequences. *Hum. Genet.* **90**: 41–54.
- Lear, A.L., Eperon, L.P., Wheatley, I.M., and Eperon, I.C. 1990. Hierarchy for 5' splice site preference determined in vivo. *J. Mol. Biol.* **211**: 103–115.
- Liu, Z.R. 2002. p68 RNA helicase is an essential human splicing factor that acts at the U1 snRNA-5' splice site duplex. *Mol. Cell. Biol.* **22**: 5443–5450.
- Lund, M. and Kjems, J. 2002. Defining a 5' splice site by functional selection in the presence and absence of U1 snRNA 5' end. *RNA* **8**: 166–179.
- Mannucci, P.M., Duga, S., and Peyvandi, F. 2004. Recessively inherited coagulation disorders. *Blood* **104**: 1243–1252.
- Margaglione, M., Santacrose, R., Colaizzo, D., Seripa, D., Vecchione, G., Lupone, M.R., De Lucia, D., Fortina, P., Grandone, E., Perricone, C., et al. 2000. A G-to-A mutation in IVS-3 of the human gamma fibrinogen gene causing afibrinogenemia due to abnormal RNA splicing. *Blood* **96**: 2501–2505.
- Michaud, S. and Reed, R. 1991. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. *Genes & Dev.* **5**: 2534–2546.
- Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A., and Steitz, J.A. 1983. The U1 small nuclear RNA-protein complex selectively binds a 5' splice site in vitro. *Cell* **33**: 509–518.
- Neugebauer, K.M., Stolk, J.A., and Roth, M.B. 1995. A conserved epitope on a subset of SR proteins defines a larger family of Pre-mRNA splicing factors. *J. Cell Biol.* **129**: 899–908.
- Reese, M.G., Eeckman, F.H., Kulp, D., and Haussler, D. 1997. Improved splice site detection in Genie. *J. Comput. Biol.* **4**: 311–323.
- Roca, X., Sachidanandam, R., and Krainer, A.R. 2003. Intrinsic differences between authentic and cryptic 5' splice sites. *Nucleic Acids Res.* **31**: 6321–6333.
- . 2005. Determinants of the inherent strength of human 5' splice sites. *RNA* **11**: 683–698.
- Sadusky, T., Newman, A.J., and Dibb, N.J. 2004. Exon junction sequences as cryptic splice sites: Implications for intron origin. *Curr. Biol.* **14**: 505–509.
- Seraphin, B. and Rosbash, M. 1989. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* **59**: 349–358.
- Seraphin, B., Kretzner, L., and Rosbash, M. 1988. A U1 snRNA:pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5' cleavage site. *EMBO J.* **7**: 2533–2538.
- Siliciano, P.G. and Guthrie, C. 1988. 5' splice site selection in yeast: Genetic alterations in base-pairing with U1 reveal additional requirements. *Genes & Dev.* **2**: 1258–1267.
- Skordis, L.A., Dunckley, M.G., Yue, B., Eperon, I.C., and Muntoni, F. 2003. Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts. *Proc. Natl. Acad. Sci.* **100**: 4114–4119.
- Spena, S., Duga, S., Asselta, R., Malcovati, M., Peyvandi, F., and Tenchini, M.L. 2002. Congenital afibrinogenemia: First identification of splicing mutations in the fibrinogen $\beta\beta$ -chain gene causing activation of cryptic splice sites. *Blood* **100**: 4478–4484.

- Stark, H., Dube, P., Luhrmann, R., and Kastner, B. 2001. Arrangement of RNA and proteins in the spliceosomal U1 small nuclear ribonucleoprotein particle. *Nature* **409**: 539–542.
- Stoltzfus, A. 2004. Molecular evolution: Introns fall into place. *Curr. Biol.* **14**: R351–R352.
- Sun, H. and Chasin, L.A. 2000. Multiple splicing defects in an intronic false exon. *Mol. Cell. Biol.* **20**: 6414–6425.
- Tarn, W.Y. and Steitz, J.A. 1994. SR proteins can compensate for the loss of U1 snRNP functions in vitro. *Genes & Dev.* **8**: 2704–2717.
- Treisman, R., Orkin, S.H., and Maniatis, T. 1983. Specific transcription and RNA splicing defects in five cloned β -thalassaemia genes. *Nature* **302**: 591–596.
- Wang, J., Smith, P.J., Krainer, A.R., and Zhang, M.Q. 2005. Distribution of SR protein exonic splicing enhancer motifs in human protein-coding genes. *Nucleic Acids Res.* **33**: 5053–5062.
- Wieringa, B., Meyer, F., Reiser, J., and Weissmann, C. 1983. Unusual splice sites revealed by mutagenic inactivation of an authentic splice site of the rabbit β -globin gene. *Nature* **301**: 38–43.
- Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* **14**: 725–731.
- Wilusz, J.E., Devanney, S.C., and Caputi, M. 2005. Chimeric peptide nucleic acid compounds modulate splicing of the *bcl-x* gene *in vitro* and *in vivo*. *Nucleic Acids Res.* **33**: 6547–6554.
- Yeo, G. and Burge, C.B. 2004. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J. Comput. Biol.* **11**: 377–394.
- Zhang, M.Q. 1998. Statistical features of human exons and their flanking regions. *Hum. Mol. Genet.* **7**: 919–932.
- Zhuang, Y. and Weiner, A.M. 1986. A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell* **46**: 827–835.

RNA

A PUBLICATION OF THE RNA SOCIETY

Cryptic splice site usage in exon 7 of the human fibrinogen B β -chain gene is regulated by a naturally silent SF2/ASF binding site within this exon

Silvia Spena, Maria Luisa Tenchini and Emanuele Buratti

RNA 2006 12: 948-958

References

This article cites 58 articles, 24 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/12/6/948.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>