An exon-specific U1 small nuclear RNA (snRNA) strategy to correct splicing defects

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A significant proportion of disease-causing mutations affect precursor-mRNA splicing, inducing skipping of the exon from the mature transcript. Using F9 exon 5, CFTR exon 12 and SMN2 exon 7 models, we characterized natural mutations associated to exon skipping in Haemophilia B, cystic fibrosis and spinal muscular atrophy (SMA), respectively, and the therapeutic splicing rescue by using U1 small nuclear RNA (snRNA). In minigene expression systems, loading of U1 snRNA by complementarity to the normal or mutated donor splice sites (5′ss) corrected the exon skipping caused by mutations at the polypyrimidine tract of the acceptor splice site, at the consensus 5′ss or at exonic regulatory elements. To improve specificity and reduce potential off-target effects, we developed U1 snRNA variants targeting non-conserved intronic sequences downstream of the 5′ss. For each gene system, we identified an exon-specific U1 snRNA (ExSpeU1) able to rescue splicing impaired by the different types of mutations. Through splicing-competent cDNA constructs, we demonstrated that the ExSpeU1-mediated splicing correction of several F9 mutations results in complete restoration of secreted functional factor IX levels. Furthermore, two ExSpeU1s for SMA improved SMN exon 7 splicing in the chromosomal context of normal cells. We propose ExSpeU1s as a novel therapeutic strategy to correct, in several human disorders, different types of splicing mutations associated with defective exon definition.

INTRODUCTION

Splicing errors represent a significant amount of disease-causing mutations. Considering only changes at canonical splice sites, ~15% of mutations were originally estimated to induce aberrant splicing (1) and similar frequencies are recorded in human disease databases [for example, 12% in cystic fibrosis (CF) and 10% in coagulation FIX (FIX) deficiency (Haemophilia B)]. However, the occurrence of splicing defects is significantly increased (~50%) when genomic variants are systematically evaluated for their effect on precursor-mRNA (pre-mRNA) processing (2,3). This unexpected effect is largely due to mutations located in non-canonical regulatory elements and highlights the difficulty in correctly predicting the consequence of genomic variants on pre-mRNA splicing (4–8).

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(9,10) or composite exonic regulatory elements of splicing (CERES) (7). The exonic elements are composed by largely degenerated sequences, overlap with the coding capacity and interact with splicing factors with a positive (SR proteins) or negative (hnRNPs) effect on the exon recognition. In general, factors associated to the splice sites and to the exonic splicing regulatory elements promote a network of interactions across the exon. As a result, the final outcome of a splicing decision depends on the equilibrium between multiple positive and negative interactions over the exon in a process called exon definition (11).

Aberrant exon skipping is a common splicing defect caused by disruption of the network of interactions that define the exon. It results from mutations at the consensus donor or acceptor sites, at the polypyrimidine tract or in the exonic regulatory elements. In general, mutations at the consensus donor site reduce the complementarity with the U1 small nuclear RNA (snRNA) (see below), substitutions at the 3'ss polypyrimidine tract interfere with the complexes assembled on the 3'ss and exonic mutations affect exonic regulatory elements.

An early event in exon definition is the recognition of the donor site by the U1 snRNP (small nuclear RiboNuclearParticle). U1 snRNP is composed by a 164 bp long U1 snRNA associated to several protein factors. To initiate splicing, the 5'ss of the U1 snRNA interacts by complementarity with the moderately conserved sequence of the 5'ss (Fig.1A). Interestingly, ~40, 22 and 5% of normal 5'ss contains, respectively, two, three or four mismatches toward the U1 snRNA (12). U1 snRNAs with a modified 5'tail that base pair exactly to the mutant donor sites have been used to correct 5'ss mutation (13–19). For example, we previously showed that a U1 snRNA complementary to the +5G/A mutant of the intron 7 donor splice site of coagulation F7 rescued splicing and protein biosynthesis and function in minigene systems (14,15). However, this correction approach appears to be limited to single mutations and, through the ability of modified U1 snRNAs to target other donor sites, it can potentially interfere with splicing of other pre-mRNAs.

Here, to study the effect of mutations on splicing and identify a correction strategy on an exon basis, we considered three donor-site substitutions and, as models for exonic mutations that affect splicing, two codon changes (A566T and Y577Y) located in the previously reported CERES element (7) (Supplementary Material, Table S1). To test their effect on splicing, the substitutions were inserted in appropriate minigenes followed by analysis of the splicing pattern in eukaryotic cells. For F9 exon 5, we prepared a novel minigene, whereas the CFTR exon 12 minigene has been previously described and extensively validated (7,8,20). Normal F9 ex 5 wt minigene showed that the exon was not completely included (~85%) in the mature mRNA (Fig. 1B, lane 3). This pattern of splicing is entirely consistent with the in vivo splicing pattern of the F9 gene in human liver, where ~90% of the exon is included (Supplementary Material, Fig. S1). The appreciable level of alternative splicing indicates that the exon 5 is poorly defined, very likely because of the presence of a weak 5'ss. In fact, it differs significantly from the consensus donor site at positions 3, 5 and 6, with a C, a U and an A, respectively (Supplementary Material, Fig. S2).

Expression studies of the F9 ex 5 mutations revealed that the majority of them induced aberrant pre-mRNA processing (Fig. 1A). The −8 and −9 transversions of the polypyrimidine tract and the mutations at the consensus 5'ss at positions −2, −1, +1 and +2 induced complete exon skipping. On the other hand, all mutations in CFTR exon 12, either located at the donor sites (in position −1, +3 and +5) or in the exon (A566T and Y577Y), induced exon skipping (Fig. 1D). In is worth noting that the A566T and Y577Y exonic variants affect exonic splicing regulatory elements (7), which are important for correct exon definition. Thus, in two model systems, several natural mutations either at the splice sites or at exonic regulatory elements induce exon skipping.

**RESULTS**

**Effects of F9 exon 5 and CFTR exon 12 mutations on pre-mRNA splicing**

We evaluated nine different natural mutations associated with Haemophilia B deficiency (Fig. 1A) and seven mutations associated to CF (Fig. 1C). The list of the mutations analysed and their associated clinical phenotype are shown in Supplementary Material, Table S1. Mutations are numbered according to their position relative to the donor or acceptor site. The mutations in the F9 gene consists of two T to G transversions at the 3'ss polypyrimidine tract and seven substitutions at or near the 5'ss of exon 5. In the CFTR exon 12, we studied five donor-site substitutions and, as models for exonic mutations that affect splicing, two codon changes (A566T and Y577Y) located in the previously reported CERES element (7) (Supplementary Material, Table S1).

**U1 snRNAs complementary to the mutant 5'ss rescue some defective donor sites**

We initially focused on mutations at donor sites, which reduce their complementarity to the 5's tail of the U1 snRNA. To understand for each mutation the role of U1 snRNA in exon recognition, we prepared U1 snRNAs expression plasmids with compensatory changes that completely restore basepairing with the wt or mutant 5'ss (Supplementary Material, Figs S2 and S3). These U1 snRNAs were cotransfected with the corresponding minigene variants followed by the analysis of the splicing pattern.

Cotransfection of U1FIXwt with the ex5 wt minigene improved the exon recognition inducing complete inclusion.
of the exon (Fig. 2A, lanes 1 and 3). Noticeably, the three synonymous changes at position −2 showed a remarkable increase in the percentage of exon inclusion after cotransfection of the corresponding complementary U1 snRNAs, with a negligible presence of aberrant transcripts (Fig. 2A, lanes 4–9). On the contrary, the splicing pattern of the other FIX mutations +1T, +1A and +2C was not affected by the corresponding complementary U1 snRNAs.

Minigene experiments with the CFTR exon 12 showed that all mutations were rescued by the corresponding complementarity U1 snRNAs. Whereas the −1T, +3C and +5A were slightly enhanced (Fig. 2B), the severe splicing defects caused by −1A and +3G mutations were efficiently corrected by the complementary U1 snRNAs, reaching ~75 and ~95% of exon inclusion. Cotransfection of U1 Cfwt with the Cfex12 wt also improved the splicing pattern (Fig. 2B, lane 3).
These results indicate that the three synonymous −2 variants in F9 exon 5 and the donor 5′ss mutations in CFTR exon 12 do not completely disrupt the 5′ss function. They produce a defective donor site, whose recognition by complementary U1 snRNA variants recovers correct exon inclusion.

U1 snRNAs complementary to intronic sequences downstream of the 5′ss correct aberrant splicing

To promote exon definition, U1 snRNP does not necessarily have to perfectly bind at the 5′ss. Some atypical 5′ss are recognized by U1 snRNA shifted by one nucleotide (21) and U1 snRNA complementary to downstream intronic sequences were originally reported to promote exon inclusion in model gene system (22,23). Thus, we tested the effect of U1 snRNA-binding downstream mutant CFTR exon 12 and F9 exon 5. For this purpose, we prepared different U1 snRNAs, whose 5′ tails were modified to base pair at variable distance downstream of the 5′ss of F9 exon 5 and CFTR exon 12 (Fig. 3). This approach was also extended to the extensively investigated SMN2 exon 7, where a weak constitutive 5′ss and a synonymous exonic substitution are associated to exon skipping (24–26). In F9, we systematically screened U1 snRNAs from position −7 till position 63 relative to the donor site junction and the analysis was performed on the ex5→2C mutant minigene. As shown in Figure 3A, all U1 snRNAs tested reduced exon 5 skipping, indicating their positive effect on exon definition. In particular fix1, fix9 and fix10 U1 snRNAs showed the strongest effect with a nearly complete rescue of aberrant splicing. In CFTR exon 12, only the U1 snRNA variant cf11 induced a significant (70%) rescue of the splicing pattern in the +3G mutant (Fig. 3D). The other U1 snRNAs had a modest effect (cf1 and cf9) or no effect (cf15 and cf33) on splicing (Fig. 3D). In the SMN2 minigene, ~20% of exon 7 is included in the final transcript, whereas cotransfection of sm2, sm17 and sm21 resulted in >80% of exon inclusion (Fig. 4B).

These results in three different gene systems indicate that U1 snRNAs binding downstream the 5′ss corrected exon skipping caused by defective 5′ss.

SMN-specific U1 snRNAs rescue exon 7 splicing in normal cells

To study the effect of different ExSpeU1s in the chromosomal context, we focussed on SMN exon 7. Normal individuals have two SMN copies, SMN1 and SMN2, that mainly differ for a synonymous substitution in exon 7. This substitution induces SMN2 exon 7 skipping and because the majority of SMA patients lack SMN1, its splicing correction is at the basis of several therapeutic attempts (27,28). To evaluate the potential therapeutic effect of SMN-specific U1s in SMA, we transfected sm17 and sm21 in HEK293 cells and evaluated the endogenous pattern of splicing. Semiquantitative analysis of splicing isoforms showed that sm17 and sm21 induced a significant increase in the percentage of SMN exon 7 inclusion. In particular, transfection of the two ExSpeU1s increased the percentage of SMN2 exon 7 from ~40 to ~70% (Fig. 5).

A unique exon-specific U1 snRNA can rescue splicing of defective splice site mutants and exonic variants

We subsequently tested the effect of the most active U1 snRNAs (fix9 for FIX and cf11 for CFTR), named exon-specific U1 snRNAs (ExSpeU1), on all splicing mutations. In F9 exon 5, fix9 ExSpeU1 completely rescued the defective donor sites of the three synonymous mutations in position −2 (Fig. 6A, lanes 5–10). At variance, the −1T, +1A and +2C 5′ss mutations, not corrected by loading the U1 snRNA directly on the
donor site (Fig. 2A), were also not influenced by ExSpeU1 fixtures (Fig. 6A, lanes 11–16). Interestingly, cotransfection of ExSpeU1 fixtures induced nearly complete exon inclusion of the two 28G and 29G intronic variants of the polypyrimidine tract (Fig. 5A, lanes 1–4). In CFTR exon 12, ExSpeU1 cf11 rescued all 5′ss mutations with different efficiency. After ExSpeU1 cf11 cotransfection, the +3G mutation showed ≏85% of exon inclusion (Fig. 6B); the percentage of exon inclusion in 21A, +3C and +5A increased to ≏50%, whereas in 21T it improved to ≏20% (Fig. 6B). Intriguingly, we observed complete splicing correction by ExSpeU1 cf11 of the two exonic A566T and Y577Y mutations (Fig. 6B, lanes 1–4). Sequencing of ExSpeU1-induced products showed correct usage of the normal intron–exon junction and no activation of cryptic splice sites. To clarify if the enhancing effect is specific for the U1 snRNA particle, we inserted the tail of two active ExSpeU1s, fix10 and cf11, in the U7 snRNA. U7 is not involved in splicing and a modified version has been extensively used to express antisense RNA sequences (29–35). The resulting U7 fix10 and U7 cf11 were cotransfected with 28G, +3G and Y577Y, respectively. In contrast to the ExSpeU1s, the corresponding U7s had no effect (Supplementary Material, Fig. S4), suggesting that U1 snRNP-specific proteins are required for splicing rescue and that ExSpeU1s are not just targeting intronic sequences with silencer function. Altogether, these data indicate that recruitment of ExSpeU1 by complementarity on intronic sequences downstream of the exon corrects multiple splicing mutations located either at 5′ or 3′ splice sites or in exonic regulatory elements. This provides a novel therapeutic strategy exploiting a unique ExSpeU1 to correct a panel of splicing defects.

Rescue of splicing by the F9 exon5-specific U1 snRNA results in FIX biosynthesis and coagulant activity

To establish whether the ExSpeU1-mediated correction of splicing of the different mutants result in a consistent rescue
of protein biosynthesis and function, we took advantage of the FIX model. FIX is a serine protease secreted from cells that can be finely monitored by protein and functional assays. The splicing mutations corrected by the ExSpeU1 fix9 (the three 5′ss variants at the 5′ss and the two transversions at the polypyrimidine tract) were tested in a splicing-competent cDNA minigene context (pBsKFIX) (Fig. 7A). In this minigene, exon 5 was inserted in FIX cDNA transcript along with part of its intronic sequences in a manner that the correction of exon skipping produces a normal transcript with secretion of a functional protein (Fig. 7A). The pBsKFIX exon 5 wt and mutant minigenes were expressed in BHK cells with or without the modified U1 snRNAs complementary to the F9 donor site (U1FIXwt) or to the downstream intronic sequence (fix9).

Probably caused by the pBsKFIX minigene context, this construct showed exon 5 inclusion in ~10% of the transcripts (Fig. 7, lane 1), while experiments with the pTB minigene (Fig. 1) and in vivo (Supplementary Material, Fig. S3) showed a ~85% inclusion. In spite of this inefficient exon 5 recognition, the secreted FIX levels in the conditioned medium were appreciable by ELISA (13 ± 1 ng/ml), western blotting (Fig. 7C) and coagulation assays (Fig. 7D). In particular, western blotting revealed the presence of a band corresponding to the full-length FIX form (~60 kDa), which migrated as plasma-derived FIX. In addition, a band corresponding to a smaller size FIX variant (~51 kDa) was also clearly detectable. This arises from translation of the in frame FIX mRNA form lacking exon 5 (Fig. 7C), leading to a FIX molecule lacking 43 amino acids of the second epidermal growth factor like domain 2 (EGF2). Notably, the mRNA splicing and western blot patterns showed remarkably different relative amount of the two isoforms, with the aberrant form predominating at the mRNA (Fig. 7B) but not at the protein (Fig. 7C) level, a finding compatible with inefficient biosynthesis of the protein without the EGF2 domain.
Transfection of the five pBsKFIX mutant minigenes showed complete skipping of the exon (Fig. 7B, lanes 2, 5, 8, 11, 14), and cotransfection of either U1FIXwt or fix9 ExSpeU1 induced a complete rescue of the splicing pattern. In all five mutants, the percentage of exon inclusion increased to level above 75% (Fig. 7B). The extent of splicing rescue mediated by these U1 snRNAs was remarkable, taking into account the inefficient exon 5 recognition in this context. Results from investigations at the protein level in the conditioned medium were consistent with those from splicing assays. The expression of the five mutants resulted in secretion of deleted FIX proteins (Fig. 7C), which did not display any appreciable coagulant activity (Fig. 7D). At variance, co-expression of variants with either U1FIXwt or fix9 ExSpeU1 induced the exclusive synthesis of the full-length FIX (Fig. 7C). This was paralleled by a complete rescue of FIX activity (Fig. 7D), which for most variants was higher than that measured for the pBsKFIX wt construct.

DISCUSSION

In this study, we provide a novel strategy to correct different types of natural splicing mutations using exon specific U1 snRNAs (ExSpeU1). ExSpeU1s bind by complementarity to intronic sequences downstream of the exon and rescue different types of splicing defects associated to exon skipping. ExSpeU1s are active on several 5′ss mutations in CFTR exon 12 and F9 exon 5, on two transversions at the polypyrimidine tract in F9 exon 5, on two exonic substitutions in CFTR exon 12 and on defective SMN2 exon 7 splicing (also due to an exonic variant). In this latter case, ExSpeU1 rescued SMN2 exon 7 splicing directly in the chromosomal context of normal cells, providing a new therapeutic strategy for SMA. On the other hand, in the model of Haemophilia B, a unique ExSpeU1 induced complete splicing correction of five different F9 mutations, thus resulting in a complete rescue of protein biosynthesis and coagulation activity.

A limited number of studies have explored the role and potential therapeutic effect of U1 snRNAs on splicing correction of donor site mutations (13–19). In all cases, the modified tails of U1 snRNA have few nucleotide changes in comparison to the WT sequences and base pair exactly to the mutant donor site. However, this mechanism seems unlikely for F9 exon 5 splicing regulatory element and its correction is at the basis of several therapeutic attempts in SMA (27,28).

Compared with classical gene replacement therapies, ExSpeU1-mediated rescue has a number of additional advantages and stimulating perspectives. The direct splicing correction maintains the regulation of the gene expression in the correct cell-specific chromosomal context under the control of endogenous transcription and pre-mRNA processing regulatory elements. The short length of the ExSpeU1s cassette (~500 bp) can also be useful in gene therapy of splicing mutations in large genes such as CFTR and F8, whose full-length transcript can represent a limiting step for their insertion in viral vectors such as AAV. On the other hand, to achieve the optimal therapeutic rescue, the expression level could be modulated in vivo by varying the number of ExSpeU1 cassette in the viral vector. In the case of dominant-negative mutations where the replacement therapy is not feasible, the splicing correction mediated by ExSpeU1 will act directly reducing the amount of the mutated toxic protein. Moreover, binding of the ExSpeU1s to intronic sequences, which are not conserved, will significantly reduce the possibility of off-target events.

Although binding of ExSpeU1s to unrelated sequences not directly involved in splicing might have an effect on other pre-mRNA processing steps (36), our result indicate that it will be possible to design ExSpeU1s with improved specificity by inserting subtle changes in sequence target and/or length. For example, ExSpeU1s sm17 and sm21 in SMN2 (Fig. 3B) or fix9, fix10 and fix13 in F9 exon 5 (Fig. 3B) bind at nearby or overlapping intronic sequences and are functionally active. Future studies in cellular and animal models will be required to address this point.

According to the classical exon definition model, a network of interactions across the exon put in contact the splicing complexes assembled on the splice sites (11,19,37). This includes the recognition of the 5′ss by the U1 snRNP, binding of U2AFs to the polypyrimidine tract and identification of exonic splicing regulatory elements by SR proteins. The mutations we have analysed here affect differently these regulatory elements and probably block splicing at the first step when the exon has to be recognized co-transcriptionally. At this stage, recruitment of U1 snRNA at the 5′ss or at downstream sequences by means of ExSpeU1s can rescue the different defects. This occurs not only for mutations at the consensus 5′ss that directly interfere with U1 snRNA binding but also for mutations located at distance from the donor site in the polypyrimidine tract or in exonic regulatory elements. In these cases, ExSpeU1s probably compensate the missing interactions and facilitate the formation of the correct network of splicing factors over the exon.

Interestingly, the two ExSpeU1s for SMN2 (sm17 and sm21) are partially complementary to a previously reported intronic splicing silencer, which binds to the negative splicing factor hnRNPA1 (38). Targeting this ISS with antisense oligonucleotides resulted in inclusion of SMN2 exon 7 in model minigenes and in cellular systems (39) and was recently shown to improve the phenotype in a SMA mouse model (27). Thus, it is possible that ExSpeU1s facilitates recruitment of the splicing machinery on the exon interfering with intronic splicing regulatory sequences located downstream the donor site. However, this mechanism seems unlikely for F9 exon 5
and CFTR exon 12, as modified U7 particles binding to the
target intronic sequences had no effect on splicing (Supple-
mental Material, Fig. S4). It is possible that ExSpeU1 acts
at different levels depending on the architecture and relative
strength of splicing regulatory elements in each exon. More
in general, ExSpeU1s-mediated induction of exon inclusion
could be used to regulate the synthesis of specific alternatively
spliced isoforms for therapeutic purposes. For example, therapeu-
sic induction of specific alternative splicing isoforms affect
tumour progression (40), angiogenesis (41,42) or aging (43),
and modified U1 snRNAs have been recently used to
promote splicing to inhibit HIV replication (44).

Even if the disease-causing splicing mutations here investi-
gated have a comparable effect on mature transcript, they
behave differently regarding their sensitivity to ExSpeU1-
mediated correction. The −1T +1T and +2C mutations in
F9 exon 5 did not respond to the ExSpeU1 fix9, whereas the
5′ss mutations in CFTR exon 12 showed a variable response
to cf11 ExSpeU1: in this case, the rescue efficiency was
optimal for the +3G, intermediate for the −1A, +3C and
+5A and low for the −1T (Fig. 5). As we have previously
suggested (19), the different rescue efficiency to ExSpeU1
indicates that donor site mutations are mechanistically differ-
ent. Interestingly, mutations that respond to ExSpeU1 (Fig. 2)
were also sensitive to U1 snRNA complementary to the
mutated 5′ss sequence (Fig. 5), suggesting a common effect
of U1 snRNAs regardless of its loading position. Non-
responsive mutations could affect the splicing progression
after the exon definition step and their rescue would require
the complementation with additional splicing factors. Even if
mutations in positions +1 and +2 are not expected to
respond to ExSpeU1 due to the obligate presence of the GT
dinucleotide at the 5′ss, the mechanism that regulates the
rescue efficiency of the other mutations is less obvious.
Some positions are known to interact with other splicing
factors in later splicing steps (like the NdeI and BglII sites
within the 3′ end site). The FIX cDNA fragments were
amplified with high-

fidelity DNA polymerase from the pCMV5-FIX vector. The
sequence from exons 1 to 4, the 5′ region, the 3′ region
and pFIX exon 5 minigene, the FIX exon 5 fragment consisting
of the last 314 bp of intron 4, exon 5 (129 bp) and the first
278 bp of intron 5 was amplified from normal genomic
DNA using FIXex5dir and FIXex5rev and cloned into the
pTB Ndel-minigene (7). Polypyrimidine tract and donor
splice site mutations were introduced in pFIXex5 between
the unique PstI and XbaI sites of pFIX exon 5 by
PCR-mediated site-directed mutagenesis. pCI-SMN2 exon 7
was obtained from Dr Adrian Krainer (CNSL, NY, USA).
Modified and exon-specific U1 snRNAs were created by re-
placing the sequence between the sites BclI and BglII with oli-
gonucleotides as previously reported (47). Sequences of
oligonucleotides are provided in Supplemental Material,
Table S2. Hybrid minigenes were verified by the sequence
analysis. Modified U7 snRNAs (U7 fix10 and U7 cf11) were
created by PCR amplification of U7SmOPT vector using
cfsh11U7 and FIXsh10U7 and Sp6 primers. PCR products
were digested with HindIII and StuI and ligated into HindIII/
StuI sites of the U7SmOPT vector and the resulting clones
verified by the sequence analysis.

The splicing-competent FIX cDNA expression cassette was
synthesized by GeneScript Inc. (Piscataway, NJ, USA) and its
sequence is available upon request. The cassette, inserted in
the pCDDA 3.1+ backbone to make the pBSK-FIX, consists
of a simian virus 40 (SV40) promoter followed by (i) the
human FIX cDNA sequence from exons 1 to 4 and the 5′
portion (544 bp) of intron 4, (ii) a unique Ndel restriction
site, (iii) the last 314 bp of intron 4, exon 5 and the 5′
portion (278 bp) of intron 5, (iv) a Ndel restriction site, (v)
the 3′ end (908 bp) of intron 5 followed by the cDNA se-
quence from exons 6 to 8, and (vi) the SV40 polyadenylation
site. The FIX cDNA fragments were amplified with high-
fidelity DNA polymerase from the pCMV5-FIX vector. The
Ndel–Ndel cassettes containing the last 314 bp of intron 4,
the exon 5 and the 5′ portion (278 bp) of intron 5 were sub-
cloned into pBSK-FIX to make the mutant variants for investi-
gation of rescue at the protein level.

Analysis of hybrid minigene expression and SMN splicing
HeLa (human cervical carcinoma), HEK393 and BHK cell
lines were grown in Dulbecco’s modified Eagle’s medium
with Glutamax I (Gibco) (DMEM with glutamine, sodium
pyruvate, pyridoxine and 4.5 g/l glucose) supplemented with
10% fetal calf serum (Euro Clone) and Antibiotic Antimycotic
(Sigma) according to the manufacturer’s instructions. HeLa
cells grown on six well plates were transfected with Effectene
reagents (Qiagen) according to the manufacturer’s protocol.
0.5 µg of hybrid minigenes were transfected either alone or
with 0.5 µg of wt/mutant U1 snRNA-encoding plasmids.
Total RNA extraction was performed after 24 h of incubation
using TRReagent (Invitrogen) and reverse transcription reac-
tion was carried out as described (47). alpha2,3 and Bra2
oligonucleotides were used for amplification of pCF exon 12
and pFIX exon 5; T7-F2 and E8-75 + 5′R oligonucleotides
for pCI-SMN2. The conditions used for the PCRs were 94°C
for 5 min for the initial denaturation, 94°C for 45 s, 56°C for
45 s, 72°C for 45 s for 35 cycles and 72°C for 10 min
for the final extension. PCR products were resolved on 2%
agarose gel electrophoresis. Quantification of exon inclusion was performed using the ImageJ software.

For SMN2 exon 7 analysis, HEK393 cells grown in six wells were transfected with 2 μg of each ExSpeU1s expression plasmid with the calcium-phosphate method and RT–PCR performed with E8-467-R and fluorescently labelled FAM-E6-F primers. Reactions were incubated at 95°C for 3 min followed by 35 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 36 s. Amplified fragments were digested with DdeI and separated on denaturing capillary electrophoresis (ABI-3100). Quantification of intensity of SMN1 and SMN2 exon 7 inclusion and exclusion bands was performed with Peakscanner™ software.

Fixation of BHK splicing-competent pBsK-FIX expression vector and reverse transcription was done as described (14). PCR reaction was carried out using pBsK-FIXdir and pBsK-FIXrev oligonucleotides. PCR products were resolved by 2% agarose gel electrophoresis. Liver RNA (First Choice Human Total RNA Survey Panel, Ambion, Inc.) was retrotranscribed in standard conditions and amplified with FIX140 and FIX279 primers.

**FIX activity and protein assays**

FIX coagulant activity was assessed by the aPTT coagulation assay (48). FIX antigen levels in the conditioned medium were determined by ELISA (Factor IX antigen, FIX; Affinity Biologicals, Ancaster, Canada). For western blotting analysis, 26 μl of the conditioned medium were incubated 5 min at 95°C and run on 4–12% SDS–PAGE (NuPAGE Bis–Tris gel, Invitrogen®, Carlsbad, CA, USA). Proteins were transferred onto a 0.2 μm nitrocellulose membrane (Whatman®, Dassel, Germany), which was blocked overnight with PBS-T and 5% low fat dry milk (Bio-Rad, Hercules, CA, USA). Membranes were then incubated for 3 h at room temperature with an anti-Human F.IX peroxidase conjugated (GAFIX-APHRP; Affinity Biologicals). The Supersignal West Femto reagent (Thermo Scientific, Rockford, IL, USA) was exploited for detection. Plasma derived FIX or rFIX-wt were used to optimize the assay.

**Supplementary Material**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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