hnRNP A1 functions with specificity in repression of SMN2 exon 7 splicing

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Homozygous deletion or mutation of the survival of motor neuron 1 gene (SMN1) causes spinal muscular atrophy. SMN1 has been duplicated in humans to create SMN2, which produces a low level of functional SMN protein. However, most SMN2 transcripts lack exon 7, resulting in a non-functional protein. A single nucleotide difference near the 5′ end of exon 7 largely accounts for SMN2 exon 7 skipping, an effect that has been attributed to loss of an exonic splicing enhancer (ESE) dependent on the SR protein splicing factor ASF/SF2 or to the creation of an exonic splicing silencer (ESS) element that functions by binding of the splicing repressor hnRNP A1. Our earlier experiments favored the latter mechanism and here we provide further evidence supporting the ESS model. We demonstrate that the striking effect of hnRNP A1 depletion on SMN2 exon 7 splicing is specific, as hnRNP A1 depletion has little or no effect on other inefficient splicing events tested, and ASF/SF2 depletion does not affect SMN1/2 splicing. By two different methods, we find a strong and specific interaction of hnRNPA1 with SMN2 exon 7 and only weak and equivalent interactions between ASF/SF2 and other SR proteins with the 5′ ends of SMN1 and SMN2 exon 7. Finally, we describe two disease-related exon-skipping mutations that create hnRNP A1 binding sites, but show that splicing can be restored only modestly or not at all by hnRNP A1 depletion. Together our results provide strong support for the idea that SMN2 exon 7 splicing is repressed by an hnRNPA1-dependent ESS, but also indicate that creation of such elements is context-dependent.

INTRODUCTION

Splicing of mRNA precursors requires specific sequences located at the ends of introns. In metazoans, these fundamental splicing signals are degenerate and often insufficient to demarcate small exons in much longer introns. Exons frequently contain positive elements known as exonic splicing enhancers (ESEs), which are most often recognized by members of the serine/arginine-rich family of splicing factors called SR proteins (1). Exon-bound SR proteins promote assembly of a large ribonucleoprotein complex called the spliceosome, which catalyzes intron removal. Predicting which exonic sequences will behave as ESEs has proved difficult for at least two reasons. First, each SR protein has rather degenerate RNA-binding specificities (2,3), and secondly, SR proteins and other positive acting factors often recognize exonic elements in a cooperative fashion, suggesting that studies of SR protein function in isolation might fail to predict their activities at particular exons in vivo (4). Adding to the complexity of exon recognition, exons may also contain splicing silencer elements (ESSs), which, in the presence of trans-acting factors that bind them, promote exon exclusion (5). Heterogeneous ribonucleoproteins (hnRNPs) typically function at ESSs, analogous to SR proteins at ESEs. The presence of ESEs and ESSs affords metazoan cells regulatory opportunities to control alternative splicing.

Exonic mutations frequently result in the skipping of exons that are normally constitutively spliced (6). Given the complexity of the exon-recognition process, determining the precise mechanism by which a single nucleotide change results in exon skipping is challenging. An example of this is seen in expression of the gene responsible for spinal muscular atrophy (SMA), an autosomal recessive neurodegenerative disease characterized by loss of lower motor neurons in the spinal cord. Homozygous loss of the survival of motor neuron 1 (SMN1) gene causes this disease (7). A nearly
identical copy of this gene, SMN2, exists at the same chromosomal location in humans (7,8). The two genes share >99% identity at the nucleotide level, and both are capable of encoding a 294 amino acid RNA binding protein, SMN. SMN is required for efficient assembly of snRNP complexes (9), and its absence leads to embryonic lethality in SMN knockout mice (10). In humans, the absence of SMN1 is partially compensated for by the SMN2 gene, which produces enough SMN protein to allow for relatively normal development in cell types other than motor neurons. SMN2 cannot fully compensate for the loss of SMN1 because, although SMN2 is transcribed at a level comparable to SMN1, a large majority of mRNAs produced by the SMN2 gene lacks exon 7. This exon 7-lacking isoform encodes a truncated, less stable protein (11). A C→T transition at position +6 of exon 7 largely explains SMN2 exon 7 exclusion (8,12), although an SMN2-specific nucleotide difference in intron 7 also contributes to full exon 7 exclusion (13).

Two models for how the C6T transition leads to exon skipping have been suggested. One model proposed by Krainer and coworkers (14,15) used an ESE score matrix (ESE finder; 16) to predict that an ESE dependent on the SR protein ASF/SF2 is present in SMN1, but disrupted in SMN2. This has been supported by in vitro splicing assays (14,17). An alternative, though theoretically compatible, model proposed creation of an ESS in SMN2 (18). Inspection of the vicinity of the C6T alteration in SMN2 revealed a sequence with significant similarity to a number of previously characterized hnRNP A1-dependent ESSs, all of which share a functionally indispensable UAG motif, which in SMN2 is created by the C6T transition. In support of this model, simultaneous depletion of hnRNP A1 and a closely related protein hnRNP A2 fully restored SMN2 exon 7 splicing in transfection assays, and hnRNP A1 was shown to bind specifically to SMN2 exon 7.

In this report, we use a variety of approaches to extend our understanding of the role of hnRNP A1/A2 proteins in exon skipping. Significantly, we show that rescue of SMN2 exon 7 splicing by hnRNP A1/A2 depletion is specific to SMN, and not due to possible global inhibitory properties of these proteins, in that siRNA-mediated depletion fails to improve splicing of two other inefficiently spliced mutant constructs. In addition, depletion of another inhibitory hnRNP protein, PTB, fails to affect SMN2 splicing, whereas it corrects the splicing of another substrate containing PTB binding sites. Additionally, using ultraviolet (UV)-crosslinking/immunoprecipitation (IP) as well as RNA affinity chromatography, we examine the binding of splicing factors to SMN1/2 exon 7 and confirm that C6T in SMN2 increases hnRNP A1 binding significantly, whereas ASF/SF2 and other SR proteins exhibit at most very weak binding to this region, in both SMN1 and SMN2. Finally, we demonstrate the creation of hnRNP A1 binding sites by two disease-related exonic mutations that cause exon skipping, Q1694X in BRCA1 and Y2113X in FBN1. However, hnRNP A1/A2 depletion fails to increase BRCA1 mutant exon inclusion and only partially rescue FBN1 splicing. Together, our data provide strong support for the hypothesis that creation of an hnRNP A1-dependent ESS plays a significant role in SMN2 exon 7 exclusion, but that formation of such elements by mutation is likely context-dependent.

RESULTS

RNAi-mediated depletion of hnRNP A1/2 but not ASF/SF2 affects SMN exon 7 splicing

We previously provided evidence that depletion of hnRNP A1 and/or 2 by siRNA treatment increased SMN2 exon 7 inclusion in HeLa cells expressing an SMN2 reporter transcript (18). To extend these results, we repeated these experiments using conditions that allow more efficient reduction in target protein levels (Fig. 1A; see Materials and Methods), to analyze the effects of hnRNP A1/2 depletion on SMN2 exon 7 splicing and conversely of ASF/SF2 depletion on SMN1 exon 7 splicing. As mentioned earlier, ASF/SF2 has been suggested to be required for SMN1 exon 7 splicing (14), but in vivo evidence for this is lacking. Consistent with our earlier results, depletion of either hnRNP A1 or hnRNP A2 resulted in partial restoration of SMN2 exon 7 inclusion, whereas their simultaneous depletion resulted in nearly full exon 7 inclusion (Fig. 1B, upper panel). In contrast, depletion of ASF/SF2 had no effect on SMN1 (or SMN2) splicing. This extends our previous results showing that splicing of the same SMN2 reporter transcript produced from a stably integrated transgene in a chicken DT40 cell line in which ASF/SF2 expression can be repressed by tetracycline was apparently unaffected following depletion of ASF/SF2 (18). Although these cells accurately recapitulate SMN1/2 exon 7 splicing, it was conceivable that the lack of an ASF/SF2 requirement might reflect the use of chicken cells. Furthermore, a caveat of the previous experiment was that it examined steady-state SMN2 mRNA levels, much of which may have accumulated prior to ASF/SF2 depletion. This was not the case in the current experiments, in which reporter transcripts were expressed only following ASF/SF2 depletion (see Materials and Methods). Taken together, these results confirm that hnRNP A1/A2 is necessary for SMN2 exon 7 exclusion, whereas ASF/SF2 is dispensable for SMN1 exon 7 inclusion.

Depletion of splicing inhibitory hnRNP proteins leads to substrate-specific effects in vivo

It has recently been argued that the effect on SMN2 splicing observed when hnRNP A1/A2 proteins were depleted was due to a global inhibitory effect of these proteins that antagonizes inefficiently spliced exons in a general manner (17,19). Although our previous data (18) established a direct correlation between hnRNP A1 binding to SMN2 exon 7 and exon exclusion, we next wished to investigate more directly the specificity of hnRNP A1/2 depletion and exon skipping.

To address this, we began by using siRNA to deplete PTB (hnRNP I), another abundant negative regulator of alternative splicing (5). PTB depletion (Fig. 1A) had no effect on the splicing of either SMN1 or SMN2 (Fig. 1B, lanes 6 and 12). PTB has been shown to inhibit splicing of a mouse IgM-based model substrate, μM-ΔE, in which a purine-rich enhancer region in the second exon has been removed. This inhibition requires the presence of one of two high-affinity binding sites within the second exon; mutation of both abrogates the repressive effects of PTB (20,21). Consistent with this, PTB depletion partially restored μM-ΔE splicing in transfected HeLa cells (Fig. 1B, lower panel, lane 10). Significantly,
however, depletion of hnRNP A1, hnRNP A2 or their combination failed to improve splicing of this substrate (Fig. 1B, lower panel, lanes 7–9). Indeed, depletion of hnRNP A1 alone actually resulted in somewhat reduced splicing of both mM and mM-DE (compare lanes 1 and 2 with 6 and 7). Although the basis for this is unknown, these results indicate that depletion of hnRNP A1/2 was unable to increase splicing of these poorly spliced substrates. These experiments provide evidence that inhibition of splicing by hnRNP A1, and PTB, is substrate-specific, and not the result of general inhibitory properties of either.

hnRNPA1 crosslinks strongly and specifically to the 5' end of SMN2 exon 7

In our previous study, we employed UV-crosslinking followed by IP to demonstrate that hnRNPA1 binds preferentially to an SMN2 RNA fragment (18). In these experiments, we used [α-32P]UTP-labeled RNA substrates. Because SMN2 contains a U at the +6 position (which is important for strong binding of hnRNPA1), although the hypothsized ASF/SF2 binding site in SMN1 is U-free (though adjacent to three 5' U's), it was suggested that biases may have arisen because of our choice of labeling nucleotide (17). To address this, we performed the same crosslinking experiments using either [α-32P]CTP and [α-32P]UTP double-labeled substrates or [α-32P]ATP-labeled substrates. We reasoned that each strategy should lead to equal labeling within the putative ASF/SF2 binding site in SMN1 (CAGACA) or the hypothsized hnRNPA1 binding site in SMN2 (UAGACA). With both labeling strategies, after incubation with HeLa nuclear extract (NE) and exposure to UV light, the SMN2 RNA preferentially crosslinked with proteins of ~24–33 kDa, which were immunoprecipitated with anti-hnRNPA1, but not anti-ASF/SF2, antibodies (Fig. 2A, compare lanes 6 and 7 with 8 and 9). In both cases, quantitation indicates that the hnRNPA1 crosslink was approximately 5-fold stronger with SMN2 than with SMN1, consistent with our earlier data (18).
These results indicate that the preferential crosslinking of hnRNP A1 to SMN2 exon 7 was not a result of bias introduced by our choice of labeling nucleotide. Notably, we did not find any evidence of an ASF/SF2 crosslink to either substrate, either using our typical conditions (10 μl NE) or when we increased the volume of NE used to 50 μl (data not shown).

The failure of ASF/SF2 to crosslink to SMN1 under any labeling condition strongly suggests that the interaction of ASF/SF2 with SMN1, if any, is very weak.

To demonstrate the specificity of the hnRNP A1 crosslink, we titrated increasing amounts of cold RNA corresponding to either an hnRNP A1 high-affinity binding site identified in our previous studies (UAGAAA; 13) or non-specific vector-derived RNA (GST-80; 22). The hnRNP A1 crosslink to SMN2 was reduced in a dose-dependent manner by the RNA corresponding to a high-affinity hnRNP A1 binding site, but not by the non-specific sequence, confirming the specificity of the interaction (Fig. 2B).

**SR proteins crosslink strongly to a shared portion of SMN1/2 exon 7 containing the Tra2-dependent ESE, but not to the exon 7 5’ end**

We next asked whether SR proteins interact with any part of SMN1/2 exon 7. Using [α-32P]ATP-labeled RNAs incubated with NE, we again performed UV-crosslinking using substrates corresponding to either the SMN1 or SMN2-specific 5’ end of exon 7, or a downstream region identical in both genes, and which contains a Tra2-dependent ESE (23). We then immunoprecipitated crosslinked proteins with two different monoclonal antibodies with pan-SR specificity, mAb1H4 and mAb104. Both antibodies detect all major members of the SR protein family, as well as the SR-related protein Tra2 (24–26). IP using mAb1H4 after crosslinking revealed no detectable bands for either the 5’ SMN1 or SMN2 specific RNA, whereas an ~40 kDa band was visible when using the shared 3’ SMN substrate (Fig. 2C, lanes 4–6). Using...
mAb104, SMN1 and SMN2 sequences both weakly crosslinked to an \(~50\) kDa protein, whereas SMN2 crosslinked weakly to an additional \(~45\) kDa protein. As with mAb1H4, mAb104 failed to IP an ASF/SF2-sized protein \((30–35\) kDa) crosslinked to SMN1. In contrast to either of the \(5'\) exon 7 probes, when the \(3'\) exon 7 RNA was used for crosslinking, mAb104 immunoprecipitated multiple strongly crosslinked proteins, the most intense of which was identical in size to that precipitated by mAb1H4 (Fig. 2C, lanes 7–9). As the crosslinking probe used in this experiment contains the previously characterized Tra2-dependent ESE, the 40 kDa crosslinked protein is likely to correspond to hTra2β, the most abundant Tra2 isoform in HeLa NE (T.K. and J.L.M., unpublished data).

The association of Tra2 and several additional SR or SR-related proteins with the SMN1/2 mid-exon ESE is characteristic of the cooperative binding of multiple splicing factors to functional ESEs. It also provides a demonstration that RNA-bound SR proteins can be crosslinked and immunoprecipitated efficiently with both pan-SR antibodies, especially mAb104. Therefore, the fact that the \(5'\) end of SMN1 exon 7 failed to crosslink substantially to proteins that can be precipitated by either antibody provides additional evidence against the presence of an ESE in the vicinity of the C6T transition.

hnRNP A1 binds tightly to immobilized SMN2 RNA whereas ASF/SF2 fails to interact strongly with SMN1

To examine more directly the factors that bind to the region of exon 7 containing the C6T transition, we used biotinylated RNA oligonucleotides which span the SMN1- or SMN2-specific nucleotide (both 18nt), or a control RNA (GST-80, 20 nt), each immobilized on streptavidin agarose beads. Each immobilized RNA was incubated with NE, washed and bound proteins were resolved by SDS–PAGE. After silver staining, specific patterns of associated proteins were observed with each RNA (Fig. 3A). Reminiscent of our UV-crosslinking experiments, a 32–33 kDa protein associated with SMN2, more weakly with SMN1, and failed to interact with the control RNA. By immunoblotting, we show that hnRNP A1 binds to these RNAs in exactly the same pattern (Fig. 3B, left panel). Quantitation of the hnRNP A1 immunoblot data indicates that 1 nmol of SMN2 RNA was capable of binding \(~20\)% of hnRNP A1 from the input, and this binding was approximately five times stronger than hnRNP A1 binding to SMN1, consistent with the previous UV-crosslinking results. ASF/SF2 association with either SMN1 or SMN2 RNA was very weak, each binding to \(<1\)% of input ASF/SF2 (Fig. 3B, right panel). In fact, SMN2 RNA appeared to bind more ASF/SF2 than SMN1 RNA; however,
as the amount of ASF/SF2 bound was so low, this variation may not be significant.

Our observation that ASF/SF2 failed to bind significantly to SMN1 RNA is at odds with recently published data, in which a similar RNA affinity approach was used (17). This inconsistency is possibly due to different ratios of RNA to NE used in the two experiments. Krainer and coworkers incubated up to 50 μg of RNA with 100 μl of NE, an amount sufficient to deplete hnRNP A1 from the supernatant using both SMN1 and SMN2 RNA. Such a large excess of RNA may be inappropriate for determining relative binding affinities. We used a much smaller ratio, /C24(6 μg of RNA in 150 μl of NE. Our results indicate that under these conditions, very little ASF/SF2 bound to either RNA. This is consistent with the siRNA and genetic experiments, indicating that ASF/SF2 plays no role in exon 7 splicing in vivo.

Disease-related exonic point mutations in BRCA1 and FBN1 that lead to exon skipping create hnRNP A1 binding sites

Having demonstrated the important role that a high-affinity hnRNP A1 binding site plays in SMN2 exon 7 exclusion, we wished to investigate whether disease-related point mutations that lead to exon skipping might share a similar mechanism of exon exclusion. The UAG motif appears to be a shared feature of many high-affinity hnRNP A1 binding sites, and we reasoned that creation of this sequence by point mutations might be a relatively common event. Some of these might represent high-affinity hnRNP A1 binding sites, and this could at least partly account for exon skipping. Alternatively, the SMN2 case, which involves cooperation with a second SMN2-specific hnRNPA1 site (13), may be unusual. We chose two mutations that cause exon skipping and also create UAG sequences. One mutation, Q1694X in the breast cancer tumor suppressor BRCA1, is a G to T transversion at position +6 in exon 18, which causes the exon to be largely excluded (27). The other, Y2113X in the fibrillin-1 (FBN1) gene, associated with Marfan syndrome, is a T to G transversion at position +26 of exon 51, which again creates a UAG sequence and causes exon skipping (28).

We prepared [α-32P]UTP-labeled short RNA corresponding to a fragment of wild-type or mutant BRCA1 exon 18 and FBN1 exon 51 and then performed UV-crosslinking in NE followed by IP with α-hnRNPA1 antibody. With the BRCA1 RNA, a prominent UV-crosslinked species was visible using the mutant but not wild-type substrate (Fig. 4A). We confirmed the identity of this species by showing that it is immunoprecipitated by the anti-hnRNPA1 antibody (Fig. 4A, lane 6). Using FBN1 RNA, we again detected a strong crosslinked species, the size of hnRNP A1, which was also immunoprecipitated by the anti-hnRNPA1 antibody. Unlike our
crosslinking experiments with SMN2 exon 7 and BRCA1, crosslinking-IP with FBN1 produced a doublet. The sequence created by this mutation is identical to the high-affinity hnRNP A1 binding site determined by SELEX (29) and also identical to an SMN2-specific intronic hnRNP A1 binding site, which also produced a doublet after IP with anti-hnRNP A1 antibodies (13). We believe this reflects binding of an additional as yet unidentified hnRNP A1 isoform.

HnRNP A1/A2 depletion has no effect on mutant BRCA1 splicing, but partially rescues mutant FBN1 splicing

Because both BRCA1 Q1694X and FBN1 Y2113X mutations create high-affinity binding sites for hnRNP A1, we next asked whether, as with SMN2, hnRNP A1 depletion might restore inclusion of the mutant exons. To test this, we constructed minigenes consisting of BRCA1 exon 17–19 (4.3 kb) and exon 50–52 of FBN1 (0.9 kb), both consisting of wild-type, the mutations described earlier, and in the case of FBN1, an additional exon 51 mutation (C6354T), which leads to exon skipping (30) but is not predicted to create an hnRNP A1 binding site. Each plasmid was transfected into HeLa cells treated with mock siRNAs or with siRNAs that result in either ASF/SF2, hnRNP A1, hnRNP A2 or hnRNP A1/A2 depletion. The BRCA1 wild-type construct efficiently produced all full-length spliced product, whereas the mutant BRCA1 transcript primarily produced the Δ18 isoform (Fig. 5A). Using ESE scoring matrices, Krainer and coworkers predicted that BRCA1 Q1694X lies within an ASF/SF2-dependent ESE, the disruption of which causes exon skipping (31). However, we show that ASF/SF2 depletion has no effect on BRCA1 wild-type splicing (Fig. 5A, lane 2). In contrast to SMN2 splicing, depletion of hnRNP A1, hnRNP A2 or the combination of the two had no effect on mutant BRCA1 splicing (Fig. 5A, lanes 8–10). Therefore, although we find no evidence of a required ASF/SF2-dependent ESE in wild-type BRCA1 exon 18, the fact that hnRNP A1/A2 knockdown failed to even partially restore exon 18 inclusion indicates that the creation of an hnRNP A1 binding site did not contribute to Q1694X exon skipping. It is therefore likely that the disruption of an ESE bound by an unknown protein is a major factor in BRCA1 Q1694X exon 18 skipping.

Results with the FBN1 constructs were distinct. Using the wild-type FBN1 construct, we observed essentially only full-length product for the mock-treated cells or for any of the depletions (Fig. 5B, lanes 1–5, note that the upper panel presents results of typical RT–PCR, whereas experiments in the bottom panel used 32P labeling to allow more accurate quantitation). With the C6354T mutant (lanes 11–15), almost complete exclusion was observed, and again none of the hnRNPA1/2 depletions had an effect. For unknown reason, ASF/SF2 depletion (lane 1) resulted in a very small increase in inclusion.
With the Y2113X mutant (lanes 6–10), we observed more limited exon 51 skipping. Partial rescue of this aberrant splicing by hnRNP A1, but not hnRNP A2, depletion was observed, whereas somewhat increased exon exclusion was induced by ASF/SF2 depletion. These results are consistent with the simultaneous disruption of an ESE, which partially impairs splicing, and the creation of a functional hnRNP A1-dependent ESS, which also contributes to exon exclusion. Experimental evidence has, in fact, suggested that the Y2113X mutation disrupts an SC35-dependent ESE (32). FBN1 again contrasts with SMN2 exon 7 splicing, in that the former cannot be fully explained by creation of an hnRNP A1 binding site. These results, together with those observed with the BRCA1 RNA, indicate not only that creation of an exonic hnRNPA1 binding site is not sufficient to form an ESS and induce exon skipping, but also provide further evidence that depletion of hnRNPA1 does not generally enhance splicing of weak introns.

**DISCUSSION**

In this article, we have extended our previous conclusions by providing additional evidence that the creation of a high-affinity hnRNP A1 binding site by the C6T transition in SMN2 is the primary cause of SMN2 exon 7 skipping. We show, in a series of UV-crosslinking/IP and RNA affinity experiments, that hnRNP A1 binding to the 5’ end of exon 7 is increased significantly by the C6T transition in SMN2. Having earlier shown that hnRNP A1/A2 depletion is sufficient to restore SMN2 exon 7 splicing (18), we show that this effect is SMN2-specific, and not the result of general inhibitory properties of these proteins that antagonize inefficiently spliced exons.

We have also provided several lines of evidence that argue against the presence of an ASF/SF2-dependent ESE at the +6 position of SMN1 exon 7. First, we extend our results with DT40 cells by showing that siRNA-mediated depletion of ASF/SF2 in HeLa cells also has no effect on SMN1 exon 7 splicing. Secondly, we show that neither ASF/SF2 nor any other SR protein binds tightly to this region in SMN1, and furthermore, the weak binding that does occur is diminished by C6T in SMN2. Thirdly, indirect support arguing against a role for ESE loss in SMN2 exon 7 skipping is provided by BRCA1 Q1694X. This mutation creates a high-affinity hnRNP A1 binding site, but does not improve exon inclusion upon hnRNPA1/A2 depletion. This result is entirely consistent with ESE loss being primarily responsible for exon skipping in this case and, in the light of the full restoration of SMN2 exon 7 splicing observed upon hnRNP A1/A2 depletion, highlights the unlikelihood of ESE loss being the primary cause of SMN2 exon 7 exclusion.

Additional evidence consistent with the existence of an SMN2-specific ESS rather than an ESE in SMN1 has recently been reported (33). Antisense oligonucleotides (ASOs) complementary to short stretches of either SMN1 or SMN2 along the length of exon 7 were tested for their effect on SMN1 and SMN2 splicing. The presence of an ASO that anneals to positions 6–20 of SMN1 had no effect on efficient exon 7 inclusion, either in vitro or in vivo. However, an ASO corresponding to the same position in SMN2 exon 7 dramatically improved exon 7 inclusion. These results are difficult to reconcile with an ESE in this region in SMN1 required for anything but put possible prevention of the binding of inhibitory factors, which could also be achieved by the ASO. We find this unlikely given that in our RNA affinity experiments, binding of any proteins to this region of SMN1 exon 7 was very weak. In contrast, these results are entirely consistent with the SMN2-specific ASO preventing binding of a repressor, such as hnRNPA1, to SMN2 exon 7.

Our starting point in the examination of factors involved in SMN1 exon 7 inclusion and SMN2 exon 7 skipping was the observation that genetic depletion of ASF/SF2 had no effect on SMN1 exon 7 inclusion (18), and this remains a compelling piece of evidence against the disruption of an ASF/SF2-dependent ESE being the primary cause of SMN2 exon 7 exclusion. Therefore, it is important that we address arguments against the conclusiveness of this experiment (17).

One argument made was that, as ESE scoring matrices detect the presence of ASF/SF2-dependent ESEs in SMN2 exon 8, the splicing of this exon might also be compromised upon ASF/SF2 depletion. As the exon 7 and exon 8 3’ splice sites are in competition, the result of this would be that exon 7 inclusion is not perceptibly affected by ASF/SF2 depletion. If loss of an ASF/SF2-dependent ESE is the primary cause of SMN2 exon 7 skipping, following this logic, ASF/SF2 depletion should at least partially restore SMN2 exon 7 splicing, as it would then be able to compete effectively with the compromised exon 8. We consistently saw no effect of ASF/SF2 depletion of SMN2 exon 7 inclusion, and we therefore believe that this proposal is unlikely. Furthermore, predictions of SR protein binding sites made by computational programs such as ESE Finder (16) are frequently inaccurate and certainly not conclusive (discussed subsequently). Another argument is that ASF/SF2 depletion may lead to upregulation of other splicing factors that may compensate for its loss in SMN1 exon 7 splicing. Superficially, this argument is not unreasonable because we and others have shown that hTra2 (α or β) overexpression can lead to increased SMN2 exon 7 inclusion (18,23). However, we find this scenario unlikely for several reasons: first, we have found by immunoblotting that ASF/SF2 depletion does not significantly alter the concentrations of other SR proteins or of hTra2 (T.K. and J.L.M., unpublished data). Secondly, as mentioned earlier, ASF/SF2 depletion did not increase SMN2 exon 7 inclusion, which indicates that compensatory upregulation of factors that can promote exon 7 inclusion, such as hTra2, did not occur. Thirdly, ASF/SF2 depletion by RNAi has been demonstrated to exert specific effects on ASF/SF2-dependent splicing (34,35). If SMN1-exon 7 splicing were ASF/SF2-dependent, then these examples suggest that an effect on exon 7 splicing should have been detected after ASF/SF2 depletion; however, this was not observed (18; this study).

The rescue of SMN2 splicing observed upon hnRNP A1/A2 depletion is supportive of our ESS model only if it is the result of inhibitory properties of these proteins that stem from sequence-specific binding. Cartegni et al. (17) suggested that hnRNPA1/A2 inhibition of SMN2 exon 7 splicing was instead the result of a general inhibitory effect of these proteins on inefficiently spliced alternative exons. One result presented as evidence for this was that hnRNPA1/A2 depletion also
improved the splicing of an SMN1 construct with an intron 6 polypyrimidine tract mutation that reduced exon 7 exclusion. Additionally, overexpression of hnRNP A1 led to a small increase in SMN1 exon 7 skipping. Finally, using an inefficient in vitro splicing system and adding large amounts of recombinant hnRNP A1, SMN1 exon 7 splicing was also susceptible to hnRNP A1 repression. Their conclusion from these experiments was that hnRNP A1 repression of SMN2 exon 7 splicing is non-specific and independent of the C6T transition. In fact, SMN1 is an inappropriate choice to test the specificity of hnRNP A1 repression, because we have shown (18: this study) that hnRNP A1 binds to SMN1 with specificity, although in the absence of mutations that weaken genuine splicing cis-elements, and at normal cellular levels of hnRNP A1, this affinity is insufficient to result in exon skipping. We have provided here two examples of inefficient splicing events where hnRNP A1/A2 depletion does not even have a small effect; one in which inhibition is mediated by another sequence-specific regulator of alternative splicing (PTB repression of μM-ΔE), and the other in which ESE disruption is apparently the primary cause of mutant exon skipping (BRCA1 Q1694X). Finally, we have shown previously that C6T alone does not fully account for exon 7 exclusion in SMN2, as SMN-intron 7 contains high-affinity hnRNP A1 binding sites, one specific to SMN2, that are required for full exon 7 exclusion (13).

The presence of a putative ASF/SF2-dependent ESE in the vicinity of the +6 position in SMN1 exon 7, as well as in BRCA1 exon 18, was hypothesized on the basis of an ESE score matrix (14). Additional experiments involved making mutations that either increase or decrease the score of ASF/SF2 around the original mutation, then testing the effect on splicing (14,31). We have shown here that the splicing of neither SMN1 exon 7 nor BRCA1 exon 18 requires ASF/SF2, indicating that loss of an ASF/SF2-dependent ESE does not account for exon exclusion in these cases. An additional example is FBN1 Y2113X, where the point mutation lowered the score only of SRp40 and SRp55 motifs (31). It was later shown that, although the mutation does fall within an ESE, the ESE is SC35-dependent and unresponsive to either SRp40 or SRp55 (32). A systematic examination of CFTR exon 9 by mutagenesis revealed a poor correlation between predictions made by ESE Finder and the exon skipping effect of mutations within that exon (36). These results highlight the importance of directly testing predictions made by programs such as ESE Finder.

Our data have shown that creation of a high-affinity exonic hnRNP A1 binding site is insufficient to create a functional ESS. In fact, our data suggest that mutations that create such sites can affect splicing by distinct mechanisms. In the case of SMN2 exon 7, the ESS created by the C6T transversion is functional because of weak splicing signals flanking the exon and because of additional hnRNP A1 sites in the downstream intron, specifically the SMN2-specific +100 site (13). As discussed earlier, our data are consistent with the idea that the sole effect of this transition is to create an ESS, that is, there is no ESE at this position in SMN1. Indeed, it is difficult to envision why an ESE would be required at this position, given the presence of the strong Tra2-dependent ESE just downstream (23). In the case of BRCA1 exon 18, the high-affinity hnRNP A1 site created appears not to function as hnRNP A1-dependent ESS, based on the lack of effect of hnRNPA1/2 depletion. We suggest that this reflects the context of this site, such as the absence of other nearby hnRNP A1 binding sites. Although it is conceivable that this site in fact creates an ESS that functions by interaction with another protein, it is perhaps more likely that the mutation indeed disrupts an ESE. The Y2113X mutation in FBN1 exon 51 suggests a third possibility. In this case, our data are consistent with the notion that an hnRNP A1-dependent ESS is created, but it is weak as only limited exon skipping is observed. Furthermore, the incomplete effect of hnRNP A1/A2 depletion is consistent with the notion that an ESE is simultaneously disrupted by the mutation. Figure 6 illustrates these three possible outcomes of exonic mutations creating hnRNP A1 binding sites.

In summary, our experiments have provided considerable experimental support for the notion that the C6T transition in SMN2 exon 7 functions by creating an hnRNP A1-dependent ESS and that the effect of hnRNP A1 is indeed sequence-specific. More generally, our data indicate that exonic mutations that create high-affinity hnRNP A1 binding sites may or may not create a functional ESS. These possibilities must be carefully evaluated when considering the effects of disease-related mutations on pre-mRNA splicing.

MATERIALS AND METHODS

Plasmids

SMN1/2 splicing constructs were described previously (13,18). BRCA1 (from exon 17 to exon 19, total 4.3 kb) and FBN1 (exon 50 to exon 52, total 957 base pair) wild-type splicing constructs were PCR-amplified and cloned by standard methods, as described previously (13,18). All point mutations, Q1694X in BRCA1 and Y2113X and C6354T in FBN1, were obtained by site-directed mutagenesis, as described previously (13,18). For short RNA probe preparation, we followed our standard short oligonucleotide cloning method described previously (13,18). All clones and mutations were verified by sequencing (GENEWIZ).

RNA interference and plasmids transfection

RNA interference was performed by a modification of a method described previously (18). Briefly, we plated 2.5–3 × 10⁶ cells per well in 24-well plates. The next day, we transfected 80 μl of lipofectamine–RNAiMAX (1μl per well, Invitrogen) mixture with 20 pmol of duplex RNAs (Dharmacon) into cells at 30–40% confluency. After cells reached semi-confluency (~90%), we transfected the cells with splicing reporter plasmids (1.0 μg plasmid per well) using Lipofectamine 2000 (Invitrogen). The next day, we collected cells for preparation of total RNAs and total cellular proteins, as described previously (18).

In vivo splicing assay

We carried out in vivo splicing assays by standard transfection/RT-PCR assays, as described previously (13,18). For radioisotope labeling quantitative RT–PCR, we used a method previously described (13).
Ultraviolet crosslinking and IP assays

We carried out UV-crosslinking and IP assays, as described previously (13,18). Antibodies used in IP were as follows: anti-HA (hemagglutinin), 12CA5 (100 μl per sample of hybridoma cells supernatant) a gift of Dr Carol Prives (Columbia University); anti-hnRNP A1 antibody 9H10 (1 μl per sample of 2.5 mg/ml, Immuquest); anti-ASF antibody 96 (5 μl per sample of 0.5 mg/ml, Invitrogen), anti-SR antibody 1H4 (6 μl per sample of 0.5 mg/ml, Invitrogen) and anti-SR antibody 104 (200 μl per sample of hybridoma cells supernatant). Protein G–agarose beads (Roche) were used for IP against mouse IgG monoclonal antibodies, but except for mouse monoclonal antibody 104 using anti-mouse IgM antibody-conjugated agarose beads (Sigma).

RNA affinity

RNA affinity purification was performed as described previously with some modifications (37). Short RNAs whose sequences correspond to the 5' end half of SMN1 and SMN2 exon 7 (18 nucleotides) and negative control GST-80 (20 nucleotides) as previously published (37,38) were obtained with 5' end biotinylation (Dharmacon). Each 1 nmol of biotinylated RNAs was bound with 100 μl of streptavidin-conjugated agarose beads (Sigma) in 500 μl of binding buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 2 M NaCl) overnight at 4°C while rotating. Beads were washed twice with binding buffer and twice with buffer D [20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT)].

NE mixture was prepared in 500 μl volume (10 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 2 mM MgCl2, 0.75 mM ATP, 25 mM creatine phosphate, 0.1 mM EDTA, 0.25 mM DTT, with 30 μg of Escherichia coli tRNA) containing 125 μl of HeLa NE and pre-incubated at 30°C for 10 min. RNA-immobilized beads were mixed with pre-incubated NE mixture and incubated at 30°C for 30 min with constant rotation. After RNA and protein binding, beads were washed with buffer D twice and buffer D without glycerol twice and eluted by adding 40 μl of protein sample buffer and boiled for 5 min. Aliquots of 8 μl of the eluted bound proteins were resolved by 10% SDS–PAGE and analyzed by western blotting with anti-hnRNP A1 antibody, clone 9H10.

Figure 6. Exonic mutations that create strong hnRNP A1 binding sites contribute to exon exclusion in diverse ways. (i) In the case of SMN1/2, a Tra2-dependent ESE (shaded box) located in the middle of exon 7 stimulates SMN1 exon 7 splicing, resulting in exon 7 inclusion. The C6T transition in SMN2 creates an hnRNP A1-dependent ESS (black box) and this induces exon 7 skipping. The activity of the SMN2 ESS is at least partly context-dependent, as a downstream intronic SMN2-specific hnRNP A1 site is required for maximal exon 7 exclusion (13). (ii) In FBN1, a T to G point mutation, Y2113X, in exon 51 disrupts a putative ESE (shaded box) and simultaneously creates an hnRNP A1-dependent ESS (black box), and these together result in exon 51 skipping. In this case, both the extent of exon exclusion and rescue by hnRNP A1 depletion were relatively weak. This suggests that high-affinity hnRNP A1 binding here produces only a modest ESS, likely reflecting context effects. (iii) In BRCA1, a G to T point mutation, Q1694X, in exon 18 disrupts an ESE (shaded box) and also creates hnRNP A1 binding site (dotted line). However, this site does not function as an hnRNP A1-dependent ESS, and disruption of an ESE appears to underlie exon 18 skipping. This example provides additional evidence that hnRNP A1 depletion does not generally activate splicing of weak exons and also highlights the importance of context in determining whether an hnRNP A1 binding site functions as an ESS. In all examples, open boxes indicate exons, thick horizontal lines indicate introns, shaded boxes indicate putative ESEs and black boxes indicate functional hnRNP A1-dependent ESSs. Small inverted triangles indicate the location of disease-related point mutations or the C6T transition in SMN1/2.

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