Muscleblind-like 1 activates insulin receptor exon 11 inclusion by enhancing U2AF65 binding and splicing of the upstream intron

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ABSTRACT

Alternative splicing regulates developmentally and tissue-specific gene expression programs, disruption of which have been implicated in numerous diseases. Muscleblind-like 1 (MBNL1) regulates splicing transitions, which are disrupted on loss of MBNL1 function in myotonic dystrophy type 1 (DM1). One such event is MBNL1-mediated activation of insulin receptor exon 11 inclusion, which requires an intronic enhancer element downstream of exon 11. The mechanism of MBNL1-mediated activation of exon inclusion is unknown. We developed an in vitro splicing assay, which robustly recapitulates MBNL1-mediated splicing activation of insulin receptor exon 11 and found that MBNL1 activates removal of the intron upstream of exon 11 upon binding its functional response element in the downstream intron. MBNL1 enhances early spliceosome assembly as evidenced by enhanced complex A formation and binding of U2 small nuclear ribonucleoprotein auxiliary factor 65 kDa subunit (U2AF65) on the upstream intron. We demonstrated that neither the 5' splice site nor exon 11 sequences are required for MBNL1-activated U2AF65 binding. Interestingly, the 5' splice site is required for MBNL1-mediated activation of upstream intron removal, although MBNL1 has no effect on U1 snRNA recruitment. These results suggest that MBNL1 directly activates binding of U2AF65 to enhance upstream intron removal to ultimately activate alternative exon inclusion.

INTRODUCTION

The majority of vertebrate genes are interrupted by introns, which are recognized and removed by the basal splicing machinery, the spliceosome, through recognition of multiple cis-acting sequence elements within the pre-mRNA. These RNA elements are recognized by RNA and protein components of the spliceosome. The mammalian spliceosome consists of five U-rich snRNA (small nuclear RNA)-containing small nuclear ribonucleoprotein (snRNP) complexes: U1, U2, U4, U5 and U6. These complexes assemble in a step-wise manner to define splice sites and ultimately catalyze intron removal and exon joining (1,2). The early (E) spliceosome complex consists of the U1 snRNP bound at the 5' splice site and U2 auxiliary factor (U2AF) bound at the polypyrimidine tract and 3' splice site. Next, the first ATP-dependent complex, complex A, forms by recruitment of the U2 snRNP to the branch site. The U4/U6.U5 tri-snRNP is then recruited, and rearrangements of RNA and protein contacts result in the removal of U1 and U4 from the complex, resulting in the formation of a catalytically activated complex. Following formation of the lariat intermediate, the 5' exon is joined with the 3' exon to form spliced mRNA and the lariat intron is degraded.

Alternative splicing is a pervasive mechanism of gene expression regulation occurring in >95% of human multi-exon genes (3–5). Alternative splicing events can be coordinately regulated by RNA-binding proteins that bind to specific motifs often located proximal to the regulated splice sites to result in developmentally and tissue-specific programs of gene expression (6). A 5' and 3' splice site may be recognized across an exon as described by the exon definition model in which assembly of splicesome complexes at one splice site enhances assembly of splicesome complexes at the splice site across the exon (7–10). A single splicing regulator can act as an activator or repressor depending on the target pre-mRNA. This context-dependent activity is thought to arise, at least in part, from the location of the regulatory cis-element relative to the regulated exon (11,12). The mechanism of splicing repression has been
characterized for several alternative splicing events. Neuro-oncological ventral antigen (Nova) impedes complex E formation by binding within the 5′ end of the intron disrupting binding of U1 snRNA to the 5′ splice site (12). hnRNP L and hnRNP A1 coordinately cause steric hindrance of U6 snRNA binding to the 5′ splice site by promoting aberrant U1 snRNA binding with exonic sequences upstream of the 5′ splice site of CD45 exon 4 (13). Polypyrimidine tract binding protein (PTB) represses c-src N1 exon inclusion by binding a portion of U1 snRNA disrupting contacts with downstream spliceosomal components, resulting in disruption of intron definition (14,15). Furthermore, PTB binds an exonic silencer and disrupts cross-exon communication by interfering with the ability of the U1 snRNP bound at the 5′ splice site to recruit U2AF65 to the upstream polypyrimidine tract (16). Muscleblind-like 1 (MBNL1) represses cardiac troponin T (cTNT) exon 5 by binding to a structured intronic silencer, which competes with U2AF65 binding to the single-stranded polypyrimidine tract (17).

Mechanisms of activated exon inclusion generally involve interactions between a splicing regulator and a protein component of early spliceosome complexes. For example, CUGBP, Elav-like family member 2 (CELF2) binds an intronic element downstream of cTNT exon 5 and activates inclusion by promoting U2 snRNP assembly through direct protein–protein interactions with two U2 snRNP components (18). T cell-restricted intracellular antigen 1 (TIA1) binds intronic enhancer elements downstream of the 5′ splice site of Fas exon 6 and interacts with the U1-C protein, stabilizing binding of the U1 snRNP to the 5′ splice site (19). This promotes U2AF65 recruitment to the polypyrimidine tract of the intron upstream of exon 6 through exon definition (16). Nova enhances spliceosome complex A formation on the downstream intron through binding intronic enhancers downstream of the alternative exon (12). Sam68 binds the upstream intron and recruits U2AF65 to the polypyrimidine tract of the CD44 V5 exon through a protein–protein interaction (20).

MBNL is a family of three CCCH-type zinc finger-containing RNA-binding proteins that regulates alternative splicing both positively and negatively during development (11,21–25). RNA sequencing, high-throughput cross-linking immunoprecipitation and microarray analysis of Mbn1 knockout mice demonstrated that Mbn1 regulates >900 splicing events in skeletal muscle, heart and brain (11,26). Loss of MBNL1 function through its sequestration on expanded CUG repeat RNA leads to pathogenic features in myotonic dystrophy type 1 (DM1) (27–30). One splicing event that is relevant to DM1 clinical features is MBNL1-mediated activation of exon 11 inclusion of the insulin receptor (IR). Adult DM1 skeletal muscle tissue shows a reversion to the embryonic splicing pattern in which IR exon 11 is predominantly skipped due to loss of MBNL1 function. Skipping of exon 11 produces a protein isoform lacking a 12 amino acid portion of the intracellular domain resulting in diminished signaling capacity and insulin insensitivity in DM1 (31,32). We previously showed that MBNL1 activates IR exon 11 inclusion by directly binding an intronic splicing enhancer element within the intron downstream of exon 11 (33,34). However, the mechanism by which MBNL1 communicates with the basal splicing machinery to activate exon inclusion is unknown.

In this study, we have characterized the mechanism by which MBNL1 activates inclusion of IR exon 11. We previously showed that a 273-nt region of the IR pre-mRNA containing exon 11 is necessary and sufficient for MBNL1-mediated regulation. MBNL1 binds a 30-nt region containing three YGCY(U/G)Y motifs that lies 93-nt downstream of IR exon 11, and mutation of this sequence disrupts MBNL1 binding and splicing activation (33). To investigate the mechanism of IR exon 11 activation, we developed an in vitro splicing assay using the minimal MBNL1-responsive region of the IR pre-mRNA and recombinant MBNL1. We show that MBNL1 binds to the intron downstream of exon 11 and promotes removal of the upstream intron. We hypothesized that MBNL1 promotes definition of exon 11 by activating assembly of spliceosome components at the splice sites flanking exon 11. Consistent with this hypothesis, we found that MBNL1 enhances spliceosome complex A on the intron upstream of exon 11 and enhances binding of U2AF65 to the polypyrimidine tract of the upstream intron. Strikingly, this response was intact regardless of whether the intron upstream of exon 11 was complete or contained only the 3′-most 52 nt, indicating that the 5′ splice site of the upstream intron is not required. The ability of MBNL1 to promote U2AF65 binding requires neither the 5′ splice site of exon 11 nor the binding of factors within exon 11, suggesting that the mechanism involves a cross-exon effect. Although MBNL1 had no effect on binding of U1 snRNA to the 5′ splice site, we demonstrated that blocking the 5′ splice site disrupts the ability of MBNL1 to activate splicing of the upstream intron. This result suggests that the 5′ splice is involved in MBNL1-mediated splicing activation downstream of U1 snRNP recruitment. This is the first characterization of the mechanism used by MBNL1 to activate alternative splicing and indicates that MBNL1 promotes inclusion of IR exon 11 by stimulating an early step of spliceosome assembly on the upstream intron.

MATERIALS AND METHODS
RNA constructs and oligonucleotide sequences
E11TNl67 contains a genomic segment of IR pre-mRNA (52 nt of the upstream intron, the entire 36 nt exon and 185 nt of the downstream intron) between chicken skeletal troponin I (sTNI) exons 6 and 7 downstream of the T7 promoter. For constructs defective in MBNL1 binding, we incorporated a mutation in the MBNL1 response element, which abolishes binding to the 30-nt RNA fragment in a gel-shift assay (33). To generate single-intron substrates, DNA templates for transcription were generated by polymerase chain reaction (PCR) from the E11TNl67 plasmid using the following primers: upstream intron substrate forward (ATGACACAGACCTGAAGC), reverse (AAG AGCAGACATGAGT), downstream intron substrate forward (TGTCTAATGAAAGTCTCC), reverse
including a 5′ splice site (GCCCAACTTACCTGTGAAC TTGCCCCCTCAGGT). The psoralen cross-linking sub-
strate was generated using forward (GGACCTAGGT ATGACTC) and reverse (CTGCTCTCCAGCACACGT ) primers. Forward primers also contained the T7
promoter sequences at the 5′-end. For the substrate in
which the 5′ splice site of exon 11 was mutated to
disrupt U1 snRNA binding, we mutated the 5′-most nu-
cleotides of the intron from GT→CG. Primers used for
reverse transcriptase (RT)-PCR were forward (GCTTCA TGCCAAGATAGA) and reverse (AGAGGTGGCTCT TTAAC). Morpholino oligonucleotides (MOs)
(GeneTools, LLC) were complementary to specific RNA
sequences: 5′ splice-site-spanning MO (5′ GGTTGCGAC AGTGAGTCTACCTA), exon 11-spanning MO (5′ G TCCCAGCGCACTGTCCTGAAGAG) and non-tar-
getting control MO (5′ ATCCATACGTAGTGGCACAC GCTGGG). The 5′ splice site MO is complementary to the last 3 nucleotides of exon 11 and the first 22 nucleo-
tides of the downstream intron. The exon 11-spanning MO is complementary to the central 25 nt of exon 11.
The polypyrimidine tract of the upstream intron was
mutated from 5′ gttccctgctcaagggtgtggtttttccacag
3′ to 5′ gAtcAACGgGcAtGaaaggcgttggAAtGgGAt
Acacag 3′ (mutated residues are capitalized).

**In vitro transcription**

DNA templates used for *in vitro* transcription of RNA
were generated either by FspI-mediated digestion of
E11TNI67 or by preparative-scale PCR using E11TNI67
as a template. The T7 promoter sequence was present in
the plasmid immediately upstream of DNA sequence to be
transcribed or was placed at the 5′-end of the forward
PCR primer. RNA was uniformly radiolabeled with
32P-UTP. Following transcription, RNA was treated
with DNaseI (Ambion) and gel isolated.

**In vitro splicing assay**

Uniformly-radiolabeled RNA was incubated under the
following conditions for *in vitro* splicing: 8 nM RNA sub-
strate, 0.8 mM dithiothreitol, 1.7 mM magnesium acetate,
1.7 mM ATP, 17 mM phospho-creatine, 1 U/μl RNAsin
(Plus; Promega) and 2.5% polyvinyl alcohol (PVA). The
percentage of the reaction comprising HeLa nuclear
extract (NE; 10.5 μg/μl, CilBiotech) for each substrate
was 27.5% for E11TNI67 and the downstream intron sub-
strate and 17.5% for the upstream intron substrate and
the isolated exon substrate. Samples with addition of re-
combinant MBL1 contained 25ng/μl of human tran-
script variant 6 protein purified from HEK293 cells
(OriGene, TP322316). Splicing reactions were conducted
in a total volume of 15 μl and were incubated for indicated
time points at 30°C. Splicing reactions were stopped by
digestion with proteinase K (Ambion) followed by RNA
purification and electrophoresis on 8 M urea 6% poly-
acrylamide gels. Gels were exposed to a film to obtain
autoradiograms or to a phosphorimager screen for quan-
tification (Typhoon Trio phosphorimager, GE
Healthcare). When splicing assays were conducted in the
presence of a MO, it was added to a final concentration
of 1 μM.

**Lariat de-branching assay**

To identify bands suspected to be lariat intermediates, we
conducted *in vitro* splicing assays, as described earlier,
puified RNA and separated RNA species by urea
PAGE. Next, we gel-isolated RNA bands and incubated
the RNA in the presence of HeLa cytoplasmic S100
extracts under the same conditions as described for the
*in vitro* splicing assay. The S100 extracts were prepared as
described (35). Reactions were incubated at 30°C for
1 h.

**Spliceosome complex formation assay**

*In vitro* splicing was carried out as described above using
the upstream intron substrate. To stop assembly, reactions
were placed on ice and heparin was added to a final con-
centration of 2 μg/μl. Reactions were incubated in the
presence of heparin at 30°C for 5 min and immediately
loaded onto 0.75-mm non-denaturing 4% acrylamide–
0.4% agarose composite gels. Gels were run at 225 V at
room temperature in 1× Tris-glycine running buffer
for 3 h.

**Ultraviolet-mediated protein–RNA cross-linking**

Splicing reactions were carried out as described earlier
except that PVA was replaced by Dignam buffer DG,
and the total reaction volume was scaled up to 30 μl.
The upstream intron RNA substrate and the isolated
exon substrate were labeled with 32P-UTP and 32P-GTP
to increase the radioactive signal. Reactions were
incubated at 30°C for 30 min and then placed as drops
onto parafilm over an aluminum block in an ice bath
placed 4 cm under an ultraviolet (UV) light. Samples
were irradiated at 254 nm for 10 min. RNase A (Sigma
Aldrich) was added to a final concentration of 1 μg/μl,
and digestions were incubated at 37°C for 30 min. Ten
percent of this sample was removed for sodium dodecyl
sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of samples before immunoprecipitation.

**Immunoprecipitation and western blotting**

For one 30 μl cross-linking reaction, 20 μl of protein
G dynabeads (Invitrogen) was combined with 3 μg of
anti-U2AF65 monoclonal antibody (clone MC3, Santa
Cruz Biotechnology) in 10 volumes of 1× binding buffer
(50 mM HEPES, pH 7.8, 100 mM NaCl, 5 mM MgOAc,
0.05% NP-40) and incubated for 1 h at room temperature
while rotating gently. Samples were added to the resin in
a total volume of 150-μl binding buffer and incubated at
room temperature for 2 h followed by 5 washes with
10 volumes of binding buffer. Bound proteins were
obtained by boiling the resin in protein loading buffer.
Following 10% SDS-PAGE, proteins were transferred to
an Immobilon-FL polyvinylidene difluoride membrane
(PVDF, Millipore) and exposed to film or a
phosphorimager screen or analyzed by western blotting.
For western blotting, U2AF65 was detected using a rabbit
polyclonal antibody (clone H-300, Santa Cruz Biotechnology) and an AlexaFluor 680-conjugated goat anti-rabbit secondary antibody (Invitrogen) for quantitative fluorescence detection. MBNL1 was detected with a mouse monoclonal antibody (Sigma Aldrich). The fluorescent signal was read and quantified by an Odyssey infrared imager (Li-Cor).

Psoralen-mediated RNA–RNA cross-linking

The RNA substrate used for psoralen cross-linking contained the T7 promoter, 5 nt of exon 11 and 130 nt of the downstream intron including the MBNL1 response element and was uniformly labeled with 32P-UTP and 32P-GTP. Before conducting a splicing assembly assay, NEs were depleted of U1 snRNA. Twenty microliters of ATP for 1 h at 30°C non-specific control oligonucleotide (36) and 1 μl of RNase H (5 U/μl, New England Biolabs) in the presence of ATP for 1 h at 30°C.

After RNase H digestion, a splicing assay was performed as described earlier including 25% HeLa NEs and 25 ng/μl AMT-psoralen (Sigma). Reactions were incubated at 30°C for 30 min and then placed as drops onto parafilm over an aluminum block in an ice bath placed 4 cm under a UV light covered with a thin glass plate. Samples were irradiated at 365 nm for 15 min. RNA was purified as described earlier and analyzed by urea PAGE followed by autoradiography.

RESULTS

MBNL1 activates inclusion of IR exon 11 in vitro

To characterize the mechanism by which MBNL1 activates splicing, we developed an in vitro splicing assay that recapitulates MBNL1-mediated activation of exon inclusion. We previously demonstrated that a human IR genomic segment containing the 36 nt exon with 52 and 185 nt of the upstream and downstream introns, respectively, is sufficient to mediate MBNL1-activated inclusion of exon 11 in vivo (33). This IR genomic segment was inserted into a chicken stTNI in vitro splicing reporter, E11TN67, such that it is flanked by two heterologous exons and flanking intronic regions (Figure 1A). This substrate contains the MBNL1 response element located 93 nt downstream of exon 11 (33). In vitro splicing of the uniformly radiolabeled E11TN67 pre-mRNA was quantified following display of products and intermediates on a denaturing urea gel (Figure 1B, quantified in Figure 1C). In addition, we conducted RT-PCR of RNA extracted from in vitro splicing reactions using primers complementary to the two flanking exons (Figure 1D, quantified in Figure 1E). The identities of the RT-PCR products are indicated and were verified by sequencing gel-isolated bands (data not shown). Using both assays, addition of recombinant human MBNL1 consistently increased exon 11 inclusion by 40 percentage points (Figure 1B–E). To determine whether activation of exon inclusion required binding of MBNL1 to the MBNL response element, we tested an identical pre-mRNA containing mutations in all three MBNL1 binding motifs within the 30-nt MBNL1 response element (Figure 1A). Specifically, each of the three MBNL1 binding motifs within this region was mutated to AUAAUA, a sequence which has been shown to abolish binding of MBNL1 to RNA (25). We previously showed that these three mutations completely abolished binding of MBNL1 in vitro and MBNL1 responsiveness in vivo (33). This mutation in E11TN67 prevented activation of exon 11 inclusion in the presence of recombinant MBNL1 (Figure 1B–E). These results indicate that MBNL1-mediated activation of IR exon 11 is robust in vitro and is mediated by MBNL1 binding to its cognate target sequence in the downstream intron.

MBNL1 promotes exon 11 inclusion by activating removal of the upstream intron

MBNL1 could promote IR exon 11 inclusion by activating removal of the downstream intron to which it is bound (consistent with intron definition), and/or by activating removal of the intron upstream of exon 11 by cross-exon communication (consistent with exon definition). To determine whether MBNL1 promotes removal of the upstream and/or downstream intron, we first analyzed the splicing intermediates of the E11TN67 pre-mRNA. MBNL1 enhanced the level of the intermediate in which the upstream, but not the downstream, intron had been removed (the 430-nt band in Figure 1B (right) and the 427-nt band in Figure 1D) indicating that MBNL1 promotes removal of the upstream intron. To directly test this, we generated two-exon pre-mRNA substrates, containing either the upstream or the downstream intron (Figure 2A). MBNL1 activated splicing of the upstream, but not the downstream, intron (Figure 2B and C). Furthermore, the MBNL1 response element was required for MBNL1-enhanced splicing of the upstream intron (Figure 2B). Importantly, mutation of the MBNL1 response element in the downstream intron substrate did not decrease the efficiency of intron removal (Figure 2C).

These results indicate that MBNL1 does not promote splicing of the intron to which it is bound. Taken together, these data reveal that MBNL1 activates exon 11 inclusion by first promoting removal of the intron upstream of exon 11. This suggests that MBNL1 promotes communication from its response element in the downstream intron, across the upstream exon, resulting in definition of the exon.

MBNL1 activates spliceosome complex a formation on the upstream intron

We next sought to determine whether MBNL1 activates an early step of spliceosome assembly on the upstream intron. The pre-mRNA containing the MBNL1-responsive upstream intron was incubated under splicing conditions, and complexes were separated using native gel electrophoresis. Over time, we saw the appearance of the ATP-dependent complex A that migrated slowly in relation to the non-specific complex H (Figure 3A). Addition of MBNL1 to the splicing reactions enhanced
formation of complex A. Importantly, the ability of MBNL1 to promote complex A formation was disrupted in the identical RNA containing the mutation of the MBNL1 response element in the downstream intron (Figure 3A and B). We observed that the activation of complex A formation by rMBNL1 was robust at 1 min and also exhibited dose-dependence (Supplementary Figure S2). We conclude that MBNL1 promotes removal of exon 11 by activating assembly of spliceosomal complex A. These results indicate that MBNL1 promotes cross-exon communication to enhance assembly of early spliceosome complexes to promote splicing of the intron upstream of exon 11.

**MBNL1 promotes binding of U2AF65 upstream of exon 11**

Our observation that MBNL1 activates assembly of complex A suggests that MBNL1 promotes assembly of early spliceosomal proteins on the pre-mRNA. We used a UV cross-linking assay on two uniformly radiolabeled IR exon 11 substrates to identify RNA–protein interactions promoted by MBNL1. One substrate was the upstream intron pre-mRNA and the other contained only exon 11.

**Figure 1.** MBNL1 activates IR exon 11 inclusion *in vitro*. (A) E11TNI67 contains a 273-nt region of the human IR pre-mRNA inserted into a sTNI splicing reporter as shown. (B) Autoradiograms of E11TNI67 RNA purified from splicing reactions and run on a denaturing gel. Two exposures of the gels are shown to visualize the exon 11 inclusion product (left) and activated appearance of the spliced upstream intron intermediate (right) by recombinant MBNL1 (rMBNL1). Mutation of the MBNL1 response element (E11TNI67 mut) disrupts activation of exon 11 inclusion. The identities of the prominent bands are based on size and sequence of cDNAs generated from isolated bands. (C) Bands in (B) were quantified using a phosphorimager, and the percentage exon inclusion was calculated as \[\frac{100 \times \text{Exon 11 inclusion signal}}{\text{Exon 11 inclusion signal} + \text{Exon 11 skipping signal}}\]. (*P < 0.05 as calculated by a two-tailed Student’s *t*-test). (D) RT-PCR performed on RNA purified from *in vitro* splicing reactions using primers in the flanking exons (shown as arrows). DNA species were analyzed by non-denaturing PAGE followed by ethidium bromide staining. The identities of the indicated bands were confirmed by sequencing. (E) Quantification of percentage exon 11 inclusion from RT-PCR products in (D) by densitometry (*P < 0.05).
52 nt of the upstream intron and 185 nt of the downstream intron (Figure 4A). For both substrates, we also tested the identical RNA containing the mutated MBNL1 response element. Following cross-linking, RNase-treated samples were separated by SDS-PAGE. By autoradiography, we observed a 42-kDa band interacting with both wild-type substrates corresponding in size to recombinant MBNL1, and this band was absent from the substrates containing the mutant MBNL1 binding sites (Figure 4A). We also saw a faint band that appeared to be enhanced by MBNL1, and that was the expected size of U2AF65 (Figure 4A). To specifically determine whether MBNL1 enhanced binding of U2AF65, we performed immunoprecipitation with a U2AF65 antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to a PVDF membrane and the level of RNA binding was quantified using a phosphorimager and normalized to the amount of U2AF65 protein detected on the same membrane by western blotting using a fluorescently labeled secondary antibody (Figure 4B). This quantitative analysis demonstrated that MBNL1 enhanced binding of U2AF65 to the upstream intron even on the substrate containing the isolated exon and partial flanking introns. MBNL1 did not affect hnRNP A1 cross-linking to the same two substrates, indicating the specificity of the U2AF65 response to MBNL1 (Supplementary Figure S3). In addition, mutation of the polypyrimidine tract or complete removal of the intron 10 segment abolished cross-linking of U2AF65 to both pre-mRNA substrates in the presence or absence of rMBNL1 (Supplementary Figure S4) demonstrating that the U2AF65 cross-link represents binding to the polypyrimidine tract of intron 10. Furthermore, enhanced binding of U2AF65 by MBNL1 is dependent on the intact MBNL1 response element in the downstream intron. These results indicate that MBNL1 promotes assembly of spliceosome complexes on an isolated exon, with flanking intronic segments consistent with the exon definition model.

To test whether MBNL1 interacts with U2AF65, we immunoprecipitated U2AF65 from HeLa NEs treated with RNase A followed by western blotting for MBNL1. We were unable to detect co-immunoprecipitation of MBNL1 with U2AF65 (Supplementary Figure S5). These data suggest that MBNL1 activates binding of U2AF65 to the upstream intron through a mechanism other than a direct and stable protein–protein interaction.
Splicing activation by MBNL1 does not require binding of factors within exon 11

We next tested whether activated splicing of the upstream intron or enhanced binding of U2AF65 to the upstream 3′ splice site by MBNL1 required assembly of a complex spanning exon 11. To accomplish this, we used an MO to block the central 25 nt of exon 11 in an in vitro splicing assay using the RNA substrate containing the upstream intron. We first confirmed that the MO bound to the RNA by demonstrating protection of 25-nt RNA fragment from RNase A digestion (Supplementary Figure S6A). Inclusion of the MO blocked progression of the splicing reaction past the first catalytic step, resulting only in accumulation of the lariat intermediate formed after cleavage at the 5′ splice site, whereas a control MO had no effect (Figure 5A, quantified in Figure 5B). The splicing inhibitory effect of antisense blockage of internal portions of a 3′ exon has been observed previously (37). Using the exon 11 blocking MO, we observe that only the first catalytic step of splicing occurs. As shown above (Figure 2B), MBNL1 activates appearance of the lariat intermediate resulting from the first catalytic step. The exon 11 MO did not disrupt the ability of MBNL1 to activate this intermediate (Figure 5A and B), confirming our earlier results that MBNL1 activates an early step of spliceosome assembly. In addition, these data demonstrate that MBNL1 activates upstream intron removal across the exon without the requirement for exon-binding factors.

Furthermore, we tested whether enhanced binding of U2AF65 by MBNL1 is affected by the exon 11 MO. We found that the exon 11 MO did not disrupt MBNL1-mediated activation of U2AF65 binding to the upstream intron using the substrate containing the complete intron or the substrate containing only the isolated exon (Figure 5C). Taken together, these data indicate that MBNL1 activates U2AF65 binding and splicing of the upstream intron independent of assembly of complexes within exon 11.

MBNL1-mediated splicing of the upstream intron requires the 5′ splice site of exon 11

The MBNL1 response element lies in the intron downstream of IR exon 11, and we have demonstrated that...
MBNL1 activates splicing of the intron upstream of IR exon 11 (Figure 2). Binding of the U1 snRNP at the 5′ splice site is known to enhance assembly of spliceosome components at the 3′ splice site upstream of the exon via exon definition (9). Therefore, we wanted to determine whether MBNL1-mediated activation of exon 11 splicing was dependent on the 5′ splice site of exon 11. We used a 5′ splice site-blocking MO to test whether the 5′ splice site of exon 11 is required for MBNL1 to activate splicing of the intron upstream of exon 11. The same concentration of a scrambled MO was used as a control. We confirmed that the 5′ splice site MO, but not the control MO, inhibited use of the exon 11 5′ splice site by demonstrating that it completely blocks splicing of the downstream intron in a two-exon substrate (Supplementary Figure S6B). When included in a splicing reaction of the upstream intron RNA, the 5′ splice site MO led to a lower basal level of splicing. This result was not surprising, as the presence of a 5′ splice site enhances removal of the upstream intron (9). While the control MO had no inhibitory effect on activation of splicing by MBNL1, the 5′ splice site MO prevented MBNL1-activated splicing of the upstream intron (Figure 6A, quantified in Figure 6B).

We next tested whether the enhanced binding of U2AF65 mediated by MBNL1 required the 5′ splice site of exon 11. We tested the effect of the 5′ splice MO on U2AF65 recruitment using both the upstream intron substrate and the isolated exon substrate. We performed UV cross-linking of each of these substrates in splicing reactions in the presence of the 5′ splice site-blocking MO. Following immunoprecipitation with a U2AF65 antibody, we observed that MBNL1 mediated activation of U2AF65 binding even in the presence of the 5′ splice site MO (Figure 6C). We confirmed this result using an RNA substrate with a mutated 5′ splice site (without using MOs) (Supplementary Figure S7). These results indicate that MBNL1 can activate binding of U2AF65 to the upstream intron using a mechanism that is independent of the 5′ splice site downstream of exon 11. Combined with the result that blocking exon 11 does not disrupt the MBNL1 response, these data indicate that MBNL1 is able to directly promote assembly of U2AF65 on the upstream intron.

To test whether the requirement for the 5′ splice site for MBNL1-activated upstream intron splicing is due to enhanced U1 snRNP binding by MBNL1, we conducted psoralen-mediated RNA–RNA cross-linking to detect binding of U1 snRNA to the pre-mRNA. We used a uniformly radiolabeled RNA substrate containing the minimal region of interest (the 5′ splice site of exon 11 and the downstream intron extending to 5 nt downstream of the MBNL1 response element) (Figure 7). This RNA was incubated under splicing conditions in the presence of cross-linking followed by immunoprecipitation for U2AF65 (as described in Figure 4B) in the presence of the exon 11 MO (E11).

Figure 5. Access to exon 11 sequences is not required for MBNL1-mediated activation of upstream intron splicing or U2AF65 binding. (A) The upstream intron RNA substrate was spliced in the presence of an exon 11-spanning (E11block) or control (ctrl) MO and visualized by autoradiography. The location of the exon 11 MO is indicated on the right. The ~240-nt band appears in some experiments using both MOs in the presence and absence of ATP, suggesting they are non-specific degradation products (data not shown). (B) Quantification of the MBNL1-induced fold change in lariat intermediate formation using the phosphorimager signal for the percentage lariat intermediate (compared with total RNA isoforms in the lane). (*P < 0.05) (C) UV
the photochemical cross-linker AMT-psoralen. Reactions were UV-cross-linked followed by RNA purification and separation by urea PAGE. NEs used in this assay were first treated with RNase H and a DNA oligonucleotide to digest U1 snRNA or a non-targeting control oligonucleotide. On digestion of U1 snRNA, we saw disappearance of a prominent cross-linked band identifying it as the pre-mRNA-U1 snRNA cross-link. However, this band did change in intensity in response to addition of MBNL1 (Figure 7). We confirmed that binding of MBNL1 and U2AF65 to IR pre-mRNA is not disrupted in the presence of psoralen in a UV cross-linking assay (data not shown). These results indicate that MBNL1 does not directly enhance U1 snRNA binding to the 5' splice site of IR exon 11. Taken together, these findings indicate that activation of IR exon 11 splicing by MBNL1 is mediated by enhanced U2AF65 binding to the upstream intron. Although the 5' splice site of exon 11 is required for MBNL1-mediated activation of upstream intron splicing, our results indicate that MBNL1 does not affect the recruitment of U1 snRNP to the 5' splice site.

DISCUSSION

MBNL1 splicing regulatory activity is context-dependent such that its influence on alternative exon inclusion depends on the pre-mRNA target. The mechanism by which MBNL1 represses splicing of cTNT exon 5 has been characterized previously (17). The work presented here represents the first characterization of the mechanism by which MBNL1 communicates with the basal splicing machinery to activate exon inclusion. We found that MBNL1 activates early spliceosome assembly across exon 11 directly on the upstream intron (Figure 2B). Specifically, MBNL1 activates U2AF65 binding and spliceosome complex A formation on the intron upstream of exon 11 (Figures 3 and 4). We confirmed that MBNL1 activates early spliceosome assembly before the first catalytic step of splicing using an exon 11-blocking MO, which prevented progression to the second catalytic step (Figure 5A). We propose that MBNL1 activates recognition of exon 11 by directly promoting assembly of complexes on intronic regions upstream of exon 11, thus ‘defining’ the exon. Exon definition can result from a mechanism in which the U1 snRNP first assembles at the 5' splice site, which subsequently recruits splicosomal complexes (U2AF65) across the exon to the upstream 3' splice site through a network of protein–protein interactions spanning the exon (9). However, several pieces of evidence indicate that MBNL1-mediated activation of U2AF65 binding is through a direct mechanism, rather than by initially activating U1 snRNP assembly at the 5' splice site of exon 11. First, MBNL1 does not enhance binding of U1 snRNA to the 5' splice site of exon 11 (Figure 7) and consequently does not activate splicing of the downstream intron to which it is bound (Figure 2C). Second, we prevented binding of factors within exon 11 using an exon-spanning MO. We did not observe disruption of MBNL1-activated upstream intron splicing or of MBNL1-activated U2AF65 binding (Figure 5). Third, we...
prevented binding of factors (such as U1 snRNP) at the 5′ splice site of exon 11 using a 5′ splice site-spanning MO. We did not observe disruption of MBNL1-activated U2AF65 binding (Figure 6C). Together, these results indicate that the ability of MBNL1 to activate binding of U2AF65 to the upstream intron does not depend on binding events at the 5′ splice site or within exon 11. We also observed that blocking the 5′ splice site of exon 11 disrupts the ability of MBNL1 to activate upstream intron splicing (Figure 6A and B). This could indicate that the mechanism activated by MBNL1 requires the 5′ splice site in steps subsequent to U1 snRNP recruitment. The direct contacts induced by MBNL1 to result in this effect are yet unknown. Important future studies will determine whether MBNL1 promotes formation of a protein complex that recruits and/or stabilizes U2AF65 on the upstream intron, although we did not observe co-immunoprecipitation of MBNL1 and U2AF65 (Supplementary Figure S5). Alternatively, MBNL1 could promote a secondary structure, which favors spliceosome assembly. In the case of cTNT exon 5 repression, MBNL1 binds a stem-loop structure at the polypyrimidine tract upstream of exon 5, which prevents U2AF65 binding to the single-stranded polypyrimidine tract (17). MBNL1 is known to bind stem-loop RNA structures (38). It is possible that MBNL1 binds to and enhances the stability of a stem-loop structure within the IR pre-mRNA promoting pre-mRNA rearrangements that enhance binding of U2AF65 to the polypyrimidine tract upstream of exon 11 (Figure 7B). Modulation of RNA secondary structure has been indicated in several other splicing regulatory mechanisms (39). It will be important to determine whether mechanistic features of MBNL1-mediated splicing activation are conserved across various RNA targets. High-throughput RNA sequencing and cross-linking immunoprecipitation-sequencing analyses revealed an ‘RNA map’ for hundreds of MBNL1-regulated splicing events (11). These studies suggested that the architecture of the RNA target impacts the splicing regulatory potential of MBNL1. Specifically, the location of the MBNL1 response element relative to the alternative exon determines whether MBNL1 will act as a splicing activator or as a repressor. Important future investigations will identify whether MBNL1 activates U2AF65 recruitment to the upstream intron through modulation of RNA secondary structure and/or a U2AF65-binding auxiliary protein complex and whether these mechanistic features are shared with other MBNL1-activated exons.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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