Heterogeneous Ribonucleoprotein M Is a Splicing Regulatory Protein That Can Enhance or Silence Splicing of Alternatively Spliced Exons*

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Splicing of fibroblast growth factor receptor 2 (FGFR2) alternative exons IIIb and IIIc is regulated by the auxiliary RNA cis-element ISE/ISS-3 that promotes splicing of exon IIIb and silencing of exon IIIc. Using RNA affinity chromatography, we have identified heterogeneous nuclear ribonucleoprotein M (hnRNP M) as a splicing regulatory factor that binds to ISE/ISS-3 in a sequence-specific manner. Overexpression of hnRNP M promoted exon IIIc skipping in a cell line that normally includes it, and association of hnRNP M with ISE/ISS-3 was shown to contribute to this splicing regulatory function. Thus hnRNP M, along with other members of the hnRNP family of RNA-binding proteins, plays a combinatorial role in regulation of FGFR2 alternative splicing. We also determined that hnRNP M can affect the splicing of several other alternatively spliced exons. This activity of hnRNP M included the ability not only to induce exon skipping but also to promote exon inclusion. This is the first report demonstrating a role for this abundant hnRNP family member in alternative splicing in mammals and suggests that this protein may broadly contribute to the fidelity of splice site recognition and alternative splicing regulation.

Alternative splicing represents an important mechanism whereby a single gene transcript can give rise to numerous spliced mRNAs, thereby greatly expanding the ribonomic and proteomic diversity that can be obtained from a limited gene number (1, 2). Despite increasing recognition that the majority of metazoan gene transcripts are subject to alternative splicing, the molecular mechanisms that regulate this process remain poorly understood. This is particularly true for alternatively spliced gene transcripts that display tightly regulated expression of distinct splice variants in different cell types or at different stages of development. The constitutive splicing process is directed by consensus sequences at the 5′ and 3′ splice sites as well as a branch point sequence generally located 20–40 nucleotides (nt) upstream of the 3′ splice site. However, these sequence determinants are not sufficient to accurately specify the actual splice sites that are used for splicing of constitutive as well as alternative exons. In recent years, numerous studies have demonstrated an important role for an additional layer of cis-acting control sequences often referred to as auxiliary cis-elements (2, 3). These sequences have been identified in both exons and introns and can have either positive or negative effects on splicing of a given exon. As such they are commonly referred to as exonic splicing enhancers or silencers and intronic splicing enhancers or silencers (ISEs or ISSs). These elements function largely through the activity of RNA-binding proteins that associate with them and influence the efficiency with which nearby splice sites are recognized by the basal splicing machinery. Most alternatively spliced exons are associated with multiple auxiliary cis-elements that can positively or negatively affect its inclusion. These observations have led to models of combinatorial control, in which the splicing outcome is determined by the net effect of several cis-elements that interact with differentially expressed RNA-binding proteins in varied cellular milieus (1, 2, 4). Although several cell-type-specific factors have previously been described, differential expression of such factors alone has not proven sufficient to account for cell-type-specific splicing decisions, which also involve contributions from ubiquitously expressed regulatory factors. Thus a challenge in the field of splicing regulation has been to determine the different contributions of numerous cis-elements and RNA-binding proteins and how their combined functions yield distinct splicing patterns in different cells.

Our studies have focused on alternative splicing of fibroblast growth factor receptor 2 (FGFR2) transcripts. Mutually exclusive splicing of two exons, IIb and IIc, gives rise to two functionally different receptors, FGFR2-IIIb and FGFR2-IIIc, in epithelial and mesenchymal cells, respectively. These exons encode the C-terminal half of an Ig-like domain in the receptor’s extracellular domain in a region that determines ligand-binding specificity. Consequently, the two different receptor isoforms display distinct binding preferences for the FGF family of ligands (5, 6). Several auxiliary cis-elements that have been shown to play a role in regulation of these alternative exons are

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2 The abbreviations used are: nt, nucleotide(s); FGFR2, fibroblast growth factor receptor 2; ISE, intronic splicing enhancer; ISS, intronic splicing silencer; hnRNP, heterogeneous ribonucleoprotein; CELF, CUG-BP and ETR-3-like factor; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; RT, reverse transcription; PPT, preprotachykinin.
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shown in Fig. 1A. Although several abundant and ubiquitously expressed factors, including polyPyrimidine tract-binding protein, TIA-1, and hnRNP A1, have been shown to bind to several of these elements and play a role in splicing regulation, the mechanism by which cell-type-specific alternative splicing of this transcript occurs has not been fully elucidated (7–14). We have focused our recent studies on an element we have termed ISE/ISS-3, so named because it has been shown to promote splicing of exon IIb (as an ISE) and at the same time to repress splicing of exon IIc (as an ISS) in epithelial cells (9, 15). Thus in cells that express FGFR2-IIib (e.g. DT3 cells) deletion of this element in transfected FGFR2 minigenes results in a partial switch from exon IIib splicing to exon IIic splicing, whereas the exclusive inclusion of exon IIic in cells that express FGFR2-IIic (e.g. AT3 or 293T cells) is unaffected by the same deletion. We have also shown that this sequence displays epithelial cell-type-specific functions in a heterologous minigene context, suggesting that it may associate with at least one regulatory factor whose expression is limited to cells expressing FGFR2-IIib (15).

In the present study we describe one of the proteins, heterogenous ribonucleoprotein M (hnRNP M), that binds directly and specifically to the wild-type ISE/ISS-3 sequence. In a cell line that normally includes exon IIic (293T), we determined that it may associate with at least one regulatory factor (e.g. AT3 or 293T cells) deletion of this element in transfected FGFR2 minigenes results in a partial switch from exon IIib splicing to exon IIic splicing, whereas the exclusive inclusion of exon IIic in cells that express FGFR2-IIic (e.g. AT3 or 293T cells) is unaffected by the same deletion. We have also shown that this sequence displays epithelial cell-type-specific functions in a heterologous minigene context, suggesting that it may associate with at least one regulatory factor whose expression is limited to cells expressing FGFR2-IIib (15).

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—The plasmids and minigenes used in this study were constructed using standard cloning techniques. pl-11- HS, pl-11- HS-CXS, and pl-11- HS-CXS-IIb Mut minigenes and their derivatives were previously described (9, 16). The plasmid minigene containing the R35 (28) exon was generated by PCR of the R35 (28) plasmid (kindly provided by Thomas Cooper) using primers TNT-Xba-F (5′-GGCCCTCTAGAGTCGAAGTCCT-3′) and TNT-Not-R (5′-TCGAGCAGAGATGGGCTTGAC-3′). This amplified a SalI to HindIII segment from the original construct that contained the 5′-end of the sequence, and the products were re-cloned at the 5′-end of the sequence, and the products were re-cloned as ClaI/XhoI fragments into the DP19RC-ΔEE plasmid. Similarly, the previously described “full-length” ISE/ISS-3, 5′-AC, and BS fragments were also inserted in pDP19RC-ΔEE (Fig. 1B). To make the construct for expression of hnRNP M, pINΔnt-hnRNP M, an EcoRV-Agel fragment from the pHCM4 construct (kindly provided by Maurice Swanson) was inserted in the previously described pIRESneo3-Δnt vector (15, 19). Generation of N-terminal FLAG-tagged hnRNP M was carried out by inserting the same EcoRV-Agel fragment into the FLAG expression vector pLNX-N-FB-B. The latter construct was derived by insertion of sequences encoding two tandem copies of the FLAG tag upstream of the multicloning site of pIRESneo3Δnt (sequence available on request). All plasmid constructs were prepared with Qiagen MidiPrep kits. Sequences were confirmed by the University of Pennsylvania Sequencing Facility.

*UV Cross-linking*—To prepare RNA probes for UV-cross-linking experiments we performed *in vitro* transcription with T7 RNA polymerase (Ambion) after digestion of the pDP-based plasmids with XhoI using the manufacturer’s recommendations. The specific activity of the 169-nt RNA transcript was 2.7 × 10^13^ cpmp/μmol. 500,000 cpmp, or 19 fmol, of [32P]UTP-radiolabeled RNA substrates were incubated at 30°C with 12–20 μg of KATO III or 293T cell nuclear extracts in a volume of 20 μl for 20 min in 1.5-ml Eppendorf tubes. The nuclear extracts from 293T cell transiently expressing the FLAG-tagged plasmids were generated by transfection of 5 μg of plasmid in 60-mm plates using TransIT-293 Transfection Reagent (Mirus) in accordance with manufacturer recommendations. 48 h after transfection, nuclear extracts were prepared following a previously described protocol (20). After incubation, the tubes were opened, placed on ice, and UV-irradiated in a Stratagene 2400 (Stratagene) at a distance of 5 cm with an energy of 6000 μJ. After addition of 2 μl of a stock RNase mix (10 μg/ml RNase A and 10 units/μl RNase T1) the samples were incubated at 37°C for 60 min. Reactions were stopped with 4× LDS sample buffer (Invitrogen) and loaded onto 10% NuPAGE Bis-Tris gel (Invitrogen). The gel was run at constant 200 V for 60–70 min, then fixed, dried, and visualized by radiography.
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Immunoprecipitation of UV Cross-linked Proteins—Immunoprecipitation of UV-cross-linked proteins was carried out following the RNase step of the UV cross-linking protocol. Precipitation of endogenous hnRNP M from KATO III nuclear extracts was performed by first pre-clearing the UV-cross-linked extracts by addition to 30 μl of pre-washed Protein G-agarose (Roche Applied Science) in 450 μl of NET-2 buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.01% Nonidet P-40, 1× protease inhibitor (Sigma)) at 4 °C and rotation for 30 min. The pre-clear supernatant was then harvested after centrifugation at 1000 × g for 1 min. 1.5 μl of hnRNP M antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the pre-cleared extract and rotated at 4 °C for 60 min and then added to another 30 μl of pre-washed protein G-agarose and incubated for an additional 60 min. The beads were then washed three times with 500 μl of NET-2 buffer, and the precipitated proteins were eluted by addition of 20 μl of 2× sample loading buffer and boiling for 5 min. The mix was transferred to a Bio-Rad microspin column, centrifuged at 500 × g for 5 min, electro-photographed on 10% NuPAGE Bis-Tris gels, and analyzed by Western blotting. Immunoprecipitation of FLAG-tagged hnRNP M from transiently transfected 293T cell nuclear extracts was performed by first pre-clearing the UV-cross-linked extracts by addition to 40 μl of pre-washed anti-FLAG M2-agarose slurry (Sigma) in 400 μl of cold NET-2 buffer. After rotation at 4 °C for 60 min, the beads were washed twice with 400 μl of NET-2 buffer and eluted by incubating for 30 min with rotation at 4 °C with 10 μg of 3× FLAG peptide in 60 μl of NET-2 buffer. The eluted proteins were harvested using Bio-Rad microspin columns and analyzed by Western as described below.

Affinity Purification—To obtain microgram quantities of unlabeled RNA for affinity chromatography in vitro transcription reactions were performed in a 50-μl volume containing 0.1 μg/μl linearized DNA template, 2 units/μl T7 RNA polymerase (Ambion), 7.5 mM NTPs, 0.1 unit/μl inorganic pyrophosphatase (Sigma), 0.4 unit/μl SUPERase-In (Ambion) in buffer containing 80 mM Hepes-KCl, pH 7.5, 24 mM MgCl2, 2 mM spermidine, and 40 mM dithiothreitol. The reaction mix was incubated overnight at 37 °C and RNA was gel-purified from 10% denaturing acrylamide gels. Pulldown assays were performed similar to previously described protocols (21, 22), with the following modifications. A 200-μl reaction mixture containing 1 nmol of RNA, 100 mM NaOAc, pH 5.0, and 20 mM sodium m-periodate (Sigma) was incubated in the dark at room temperature for 1 h. The oxidized RNA was precipitated with ethanol and resuspended in 500 μl of 100 mM NaOAc, pH 5.0. 200 μl of adinic acid dehydrazide bead slurry (100 μl of packed beads, Sigma) were washed twice with 1 ml of 100 mM NaOAc, pH 5.0. The resuspended RNA was added to the beads and rotated overnight at 4 °C. The beads with bound RNA were then washed twice with 1 ml of 2 mM NaCl and three times with 1 ml of Buffer D (23) freshly supplemented with 0.5 mM dithiothreitol and 1× phenylethylsulfonyl fluoride. A 1-ml final volume of a mix containing 500 μl of KATO III nuclear extract in the presence of 1.6 mM MgCl2, and 5 mg/ml heparin was added to the washed beads. After rotating for 30 min at room temperature the beads were washed four times with 1 ml of buffer D supplemented with 2 mM MgCl2 and 75 μg/ml hepa-rin. The proteins were eluted by addition of 60 μl of 1.5× SDS loading buffer and heating at 90 °C for 5 min. Beads and buffer were transferred to Handee spin columns (Pierce) and centrifuged at 1000 × g for 1 min. Eluted samples were loaded on 4–12% NuPAGE Bis-Tris gel (Invitrogen). The gel was stained using either SilverSNAP (Pierce) or Colloidal Blue staining kit (Invitrogen), and the bands of interest were excised and submitted for analysis using tandem mass spectrometry performed by the University of Pennsylvania Proteomics Core Facility.

Western Blotting—To detect antibodies against hnRNP M we used the standard technique described previously (24), with modifications. The nitrocellulose filter was blocked with 2.5% Carnation milk overnight at 4 °C. The primary antibody was used at a 1:200 dilution for mouse monoclonal hnRNP M1–4 (Santa Cruz Biotechnology) or 1:1,000 dilution for monoclonal anti-FLAG M2 (Sigma). The secondary anti-mouse Ig, horseradish peroxidase-linked whole antibody (Amersham Biosciences) was used at a 1:10,000 dilution.

Cell Culture, Transfection, RNA Purification, and RT-PCR Analysis—These procedures were performed as previously described (9). For transient co-transfections, 293T cells were co-transfected in 12-well plates with 2 μg of total amount of plasmid (1 μg of a minigene and either 0.3, 0.5, or 1.0 μg of pIN-ΔInt-hnRNP M, normalized to 1 μg with empty vector using Lipofectamine 2000 (Invitrogen)). RNA was harvested 48 h after transfection. RNA from stably transfected AT3 and DT3 cells was prepared after 2 weeks of growth in selective media. Quantification was performed using a Molecular Dynamics PhosphorImager. Primers pl-11(-H3)-F and P11-P11-R (16) were used to assay for inclusion or skipping of exon IIIc and exon R35 (28) in pl-11-FS, pl-11-FS-CXS-Illb-Mut, and pl-11-R35 (28) constructs and T7 (5′-TAATTACGACT- CACTATAGG-3′) and Rat-PPT-Ex5-R (5′-GTGAGAGATC-TGACCATGCC-3′) primers for assay of inclusion or skipping of PPT exon 4 in the pPPT-106 construct. Molar amounts of final products where alternatively spliced exons were either included or skipped were normalized using exon included/exon excluded ratios: 1:2.05 for pl-11-FS and pl-11-FS-CXS-Illb Mut constructs; 1:1.21 for pl-XN-R35 (28); and 1:1.23 for pPPT-106.

RESULTS

Identification of Sequences in the 5′ Half of ISE/ISS-3 That Are Sufficient to Promote Exon IIIb Inclusion and Exon IIIc Skipping in Cells That Express FGFR2-IIb—Using stable transfection minigene assays, we previously carried out extensive mutational analysis of an 85-nt sequence constituting ISE/ISS-3 and determined that mutations or deletions within the 5′-end of the sequence were more detrimental to its function than alterations at the 3′-end (Fig. 1B) (9, 15). These experiments were carried out using an FGFR2 minigene, pl-11-FS-CXS, that contains both exons IIIb and IIIc and their flanking introns, but in which ISE/ISS-3 is deleted and replaced by two convenient restriction enzymes (Fig. 2A). Transfection of the DT3 cell line, which expresses endogenous FGFR2-IIb, with pl-11-FS-CXS demonstrated predominant splicing of exon IIIc (Fig. 2B, lanes 1–3). Analysis of results with these minigenes, performed by RT-PCR using primers complementary to sequences in the exons flanking exons Illb and IIIc, revealed a product of 386 or 383 bp.
depending on whether exon IIIb or exon IIIc was spliced, respectively. Because this size difference is difficult to distinguish, we digest these RT-PCR products separately with AvaI or HincII, which digest products containing exon IIIb or IIIc, respectively. Comparison of the fraction of products digested with these enzymes can therefore be used to determine the percentage of products that contain exon IIIb or IIIc. Transfection of a minigene in which ISE/ISS-3 has been restored to its normal position results in predominant splicing of exon IIIb and skipping of exon IIIc (Fig. 2B, lanes 4–6). Insertion of three tandem copies of the 43 nt that comprise the 5′/H11032 half of ISE/ISS-3 (3′/H11003 WT) (Fig. 1C) likewise restores predominant exon IIIb inclusion (Fig. 2B, lanes 7–9). However, insertion of the same tandem copies containing the GU to AC mutations shown in Fig. 1C (3′/H11003 MT) yields predominant exon IIIc splicing in transfected DT3 cells (Fig. 2B, lanes 10–12). Importantly, these results demonstrate the function of ISE/ISS-3 in promoting exon IIIb inclusion and exon IIIc skipping are only observed in cells that express endogenous FGFR2-IIIb. Transfection of these minigenes into cells that express endogenous FGFR2-IIIc (e.g. AT3 and 293T) results in exclusive splicing of exon IIIc, and this outcome is observed in either the presence or absence of ISE/ISS-3 (data not shown).

Identification of hnRNP M as an ISE/ISS-3-binding Protein—Given the important role of ISE/ISS-3 in mediating cell-type-specific splicing of FGFR2 exon IIIb in lieu of exon IIIc, we carried out biochemical assays to identify factors that bound specifically to this regulatory element. To characterize protein factors that bind ISE/ISS-3 we initially carried out in vitro UV-cross-linking studies using radiolabeled RNAs containing the three tandem copies of the 5′/H11032 half of ISE/ISS-3 (3′/H11003 WT), the same tandem copies containing mutations (3′/H11003 MT), or unrelated control RNA sequences. For our studies, we used nuclear extracts prepared from a cell line, KATO III, that also expresses FGFR2-IIIb as such extracts are expected to express the full complement of factors required for expression of this isoform, including potential cell-type-specific factors (9). After UV-cross-linking and RNase digestion, SDS-PAGE analysis was carried out to identify the molecular masses of proteins that cross-linked to the 3′/WT RNA (Fig. 3A). Several proteins that cross-linked specifically to the 3′/WT RNA were identified. These included bands of 110, 80, 70, 55, 50, and 20 kDa. To identify these proteins, we performed RNA affinity chromatography by covalently linking the same RNAs to adipic acid dihydrazide-agarose beads. After binding with KATO III nuclear extracts, the beads were...
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hnRNP M binds specifically to ISE/ISS-3—Although the results using RNA affinity columns showed that hnRNP M bound specifically to ISE/ISS-3 as well as the 3×WT sequence, these results are not sufficient to prove that the interaction is direct. It is possible that hnRNP M may interact with ISE/ISS-3 indirectly through another RNA-binding protein. Furthermore, given the abundance of hnRNP proteins in nuclear RNA complexes, an indirect association via another protein could also be mediated by RNAs bound by both proteins. Because RNA-protein cross-linking with ultraviolet light is accepted as evidence of a direct association, we immunoprecipitated the cross-linked extracts with anti-hnRNP M antibodies to determine whether hnRNP M was indeed one of the proteins seen by direct UV-cross-linking (27). Immunoprecipitation of proteins cross-linked to wild-type ISE/ISS-3 (WT) in KATO III nuclear extracts with the monoclonal hnRNP M antibody revealed a band that corresponded in size to hnRNP M and one of the proteins seen by direct UV-cross-linking (Fig. 4, lanes 1 and 3). This immunoprecipitated band was not observed with the 5′-AC mutant sequence (5′-AC) or with uncoupled protein G beads (Fig. 4, lanes 4–6). However, because immunoprecipitation with the hnRNP M antibody was very inefficient, this band was faint even after prolonged film exposure. For this reason we contrast, analysis from the mutant eluates identified only 28 peptides that matched hnRNP M.

To confirm binding of hnRNP M to the full-length ISE/ISS-3, we performed Western immunoblotting of proteins bound to this RNA sequence using hnRNP M monoclonal antibodies (25). These studies confirmed that greater quantities of hnRNP M were present in eluates from the column containing the 3×WT RNA than the 3×MT RNA and no binding to the beads alone could be detected (Fig. 3C, lanes 2–4). Furthermore, hnRNP M binding to the full-length ISE/ISS-3 sequence was equivalent to that observed with the 3×WT sequence (Fig. 3C, lanes 1 and 2). Of note, these antibodies recognized four protein bands representing hnRNP M. These bands (M1–M4) appear to represent different alternatively spliced isoforms of the protein (25). We also performed Western immunoblotting using eluates from the full-length ISE/ISS-3 together with another previously described mutant, 5′-AC, as well as an unrelated size-matched RNA, BS, that lacks splicing regulatory function (15). Once again, hnRNP M binding to the mutant or to the control RNA was significantly less than that observed with the wild-type element (Fig. 3D, lanes 1–3). Interestingly, hnRNP M was previously shown to bind to poly(G) and poly(U), but not poly(A) or poly(C), homopolymers (25, 26). Our results are therefore consistent with G- and U-rich sequences in the 5′-end of ISE/ISS-3 as comprising a site for hnRNP M binding that is disrupted when substituted by A and C nucleotides.

**hnRNP M Binds Directly to ISE/ISS-3**

hnRNP M binds directly to ISE/ISS-3 RNA immobilized on adipic dihydrazide-agarose beads. Regions of the gel that were excised and submitted for tandem mass spectrometry were several proteins in the 70- to 80-kDa size range. This region of the gel was excised and submitted for tandem mass spectrometry (Fig. 3B). To further characterize those proteins that specifically bound the wild-type sequence, the same region of the gel containing proteins bound to the mutant control RNA was also excised and analyzed. Mass spectrometry analysis identified hnRNP M as the protein that matched the greatest number of peptides from the wild-type sequence with a total of 239 peptides that could be matched to the protein sequence. In contrast, analysis from the mutant eluates identified only 28 peptides that matched hnRNP M.

**FIGURE 2. Tandem repeats of the 5′ half of ISE/ISS-3 mediate cell-type-specific activation of exon IIb and silencing of exon IIc.** A, schematic of the pl-11-FS-CXS minigene. Boxes represent exons, and solid lines represent introns. Alternatively spliced exons with their sizes (in nt) are indicated as shaded boxes. The ClaI and Xhol sites in the parental vector replace ISE/ISS-3, and this plasmid version is thus designated “No insert.” U and D indicate heterologous adenoviral exons upstream and downstream of exons IIb and IIc, respectively. The 85-nt full-length ISE/ISS-3, 3×WT, or 3×MT sequences were inserted as indicated between the ClaI and Xhol sites. B, results of RT-PCR of RNA from DT3 cells stably transfected with the indicated minigenes. U, undigested product; A, product digested with AvaI; H, product digested with HincII, shown above each lane. M, molecular weight markers. The RT-PCR products that contain either exon IIb or exon IIc (U-B/C-D) or exclude these exons (U-D) are schematized at the right. The percent exon IIb inclusion is indicated beneath results for each minigene. Quantification represents the percentage of products that include exon IIb (the band remaining after HincII digestion) divided by the sum of these products and products, including exon IIc (the band remaining after AvaI digestion).

**FIGURE 3. HnRNP M binds specifically to the wild-type ISE/ISS-3 sequence.** A, autoradiograph of SDS-PAGE gel with RNA-protein complexes resulting from UV cross-linking. M, molecular weight markers. B, silver-stained gel demonstrating proteins eluted from RNA immobilized on adipic acid dihydrazide-agarose beads. Regions of the gel that were excised and submitted for mass spectrometry are boxed. C, HnRNP M binds efficiently to wild-type ISE/ISS-3 (WT) and the 3×WT RNA, but not to the 3×MT RNA or beads alone (B). Western blotting of proteins eluted from the indicated immobilized RNAs with anti-hnRNP M antibodies. D, HnRNP M does not bind to a second mutant control RNA (5′-AC) or to the unrelated BS RNA.
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FIGURE 4. *hnRNP M binds directly to ISE/ISS-3.* **A,** UV-cross-linking results for the wild-type (WT) or 5′-AC mutant RNA are shown in lanes 1 and 2. The arrow indicates an ~80-kDa band believed to represent hnRNP M. The cross-linked extracts were immunoprecipitated with mouse monoclonal hnRNP M antibodies (lanes 3 and 4) or protein G beads alone (lanes 5 and 6). The position of a cross-linked ~80 kDa band that was only immunoprecipitated with the WT RNA, and the hnRNP M antibody is indicated by the arrow. **B,** nuclear extracts from 293T cells transiently transfected with either the empty FLAG vector (FLAG-EV) or FLAG-tagged hnRNP M (FLAG-hnRNP M) were analyzed by Western immunoblotting with either anti-FLAG or anti-hnRNP M antibodies. Note that the FLAG-tagged protein has slower mobility that distinguishes it from endogenous hnRNP M and that the protein levels of each are roughly similar. **C,** Western blotting of 293T nuclear extracts following immunoprecipitation with anti-FLAG affinity resin. Immunoprecipitated material is designated “pellet” (lanes 1 and 2) and indicates material bound to the column and eluted with 3× FLAG peptide after column washes. Supernatants (SN) of unbound proteins are in lanes 3 and 4. **D,** UV-cross-linking results in 293T cells transfected with the indicated plasmids and incubated with the WT or 5′-AC RNAs (lanes 1–4). The arrow indicates an ~80 kDa cross-linked band seen only with the WT RNA and which is also more evident in extracts transfected with FLAG-tagged hnRNP M. A cross-linked band of the same mobility (lane 5) is only seen following immunoprecipitation with anti-FLAG affinity resin from cells transfected with FLAG-tagged hnRNP M (and not the empty FLAG vector control) and only with the wild-type RNA.

These results support a direct and sequence-specific interaction between hnRNP M and ISE/ISS-3.

### hnRNP M Binding to ISE/ISS-3 Plays a Role in FGFR2 Exon IICc Silencing

—Given the dual role of ISE/ISS-3 in inducing exon IIBb inclusion and exon IICc skipping, we first performed transient co-transfection experiments using plasmids expressing the cDNA for hnRNP M and an FGFR2 reporter minigene, pl-11-FS, that contains both exons IIB and IIC as well as wild-type ISE/ISS-3 (Fig. 5A). For these experiments we used 293T cells, because these cells have been shown to express endogenous FGFR2-IICc, but to show an increase in exon IIBb splicing and a decrease in exon IICc splicing in response to exogenous overexpression of splicing factors, such as TIA-1 or Fox proteins (10, 11, 13, 19). When the FGFR2 minigene was co-transfected with the empty expression vector and analyzed by RT-PCR using primers complementary to sequences in the exons flanking exons IIB and IIC, we noted a single 283-bp band that represented nearly exclusive splicing of exon IICc (Fig. 5B, lanes 1–3). The same band was also observed when we transfected the same plasmid expressing increasing amounts of hnRNP M, and again the product represented nearly exclusive splicing of exon IICc (Fig. 5B, lanes 4–9, U-B/C-D product). However, in this case we noted a dose-dependent increase in the amount of an RT-PCR product of 138 bp, which we have previously determined to represent a product in which neither exon IIBb or IICc is spliced, but the flanking exons are directed ligated (Fig. 5B, U-D product). This result suggested that, although hnRNP M binds specifically to the wild-type ISE/ISS-3 sequence and plays a role in exon IICc silencing, other ISE/ISS-3-binding
proteins are required to carry out its role in activating exon IIIb splicing.

To further determine the role of ISE/ISS-3 in mediating the function of hnRNP M in silencing exon IIIc, we performed co-transfections using FGFR2 minigenes that contained the wild-type ISE/ISS-3, or control minigenes that did not. To simplify our analysis, we used a previously described minigene, pI-11-FS-CXS-IIIb Mut, in which the splice sites flanking exon IIIb were mutated to preclude its splicing, thus facilitating more direct analysis of exon IIIc inclusion versus skipping (Fig. 6A) (9, 15). This construct was used to insert the same full-length ISE/ISS-3 element contained the 5′-AC mutations or was replaced by the unrelated control sequence (Fig. 6B and C, lanes 5–8). Thus, these experiments demonstrated that hnRNP M binding to ISE/ISS-3 promotes exon IIIc skipping, but also strongly suggested that this effect on exon silencing also involves binding at other sites flanking this alternative exon. We did note that the baseline level of exon IIIc silencing obtained with the 5′/H1032-AC mutant sequence was somewhat higher than that with the other minigenes. This may reflect some serendipitous effect of inserting a “new” sequence in this position and is a frequent issue that can arise when engineering sequence alterations into minigenes as

FIGURE 5. hnRNP M promotes exon IIIc silencing in 293T cells co-transfected with an FGFR2 minigene. A, schematic of FGFR2 minigene pI-11-FS containing both exon IIIB and IIIC, as well as all relevant flanking introns sequences (including ISE/ISS-3). U, upstream adenoviral exon; D, downstream adenoviral exon. Arrows indicate the positions of primers used for RT-PCR quantification. B, RT-PCR analysis of 293T cells co-transfected with pI-11-FS and a plasmid containing the coding sequence for hnRNP M. U, undigested product; A, product digested with Aval; H, product digested with HincII, shown above each lane. M, molecular weight markers. Cells were transfected with the indicated amount of plasmid containing hnRNP M of the empty vector control (EV). The RT-PCR products that contain either exon IIIB or exon IIIC (U-B/C-D) or exclude these exons (U-D) are schematized at the right. The percent exon IIIC skipping is indicated beneath the lanes representing results of each co-transfection. In this and subsequent co-transfections, the percent exon skipping represents the average from three experiments and the standard deviations are indicated.

FIGURE 6. ISE/ISS-3 contributes to the efficiency with which hnRNP M can induce exon IIIc silencing. A, schematic of the pl-11-FS-CXS-IIIb Mut minigene in which the 5′ and 3′ splice sites of exon IIIB have been mutated to preclude exon IIIB splicing. Splice site mutations are denoted by an X, and the hatched box indicates where the ISE/ISS-3 sequence or mutant controls were inserted. The white arrow indicates the silencing function of ISE/ISS-3 on exon IIIC. B, RT-PCR analysis of 293T cells transfected with the minigenes containing the indicated insert and either the empty vector control (EV, lanes 1, 3, 5, and 7) or the vector expressing hnRNP M (lanes 2, 4, 6, and 8). Cells were transfected with 1.0 μg of the respective minigene and 0.3 μg of the hnRNP M expression plasmid. The products representing either inclusion (U-C-D) or skipping (U-D) of exon IIIC are indicated at the right. C, graphical representation of the results shown in B.
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FIGURE 7. hnRNP M promotes silencing of a preprotachykinin (PPT) exon and inclusion of a troponin-derived exon. A, schematic of the pPPT minigene in which the PPT exon and flanking intronic sequences are present between exons 3 and 5 of FGFR1. The alternatively spliced PPT exon 4 is represented by the shaded box. B, RT-PCR analysis of 293T cells co-transfected with the minigene and the indicated amounts of the hnRNP M expressing plasmid. The percent exon 4 skipping is presented below each lane. C, schematic of the troponin-derived pl-XN-R35 (28) minigene. D, RT-PCR analysis of 293T cells co-transfected with the minigene and the indicated amounts of the hnRNP M-expressing plasmid. The percent exon R35 (28) inclusion is presented below each lane.

close-specific intronic splicing enhancer elements (17, 18). As shown in Fig. 7B, co-transfection of a minigene containing the R35 (28) exon with the hnRNP M construct revealed a dose-dependent increase in the level of exon inclusion (note that the results are shown here as exon inclusion). Thus, our results suggest that hnRNP M can affect the splicing of a number of alternatively spliced exons, consistent with a broader role in global splicing regulation. Interestingly, however, depending on the context in which it is studied, this factor can either positively or negatively influence exon definition.

DISCUSSION

We have identified hnRNP M as a factor that binds directly and specifically to the ISE/ISS-3 auxiliary cis-element. We have previously shown that this element functions to regulate splicing only in cell types that express FGFR2-IIIb and that the element functions both to activate splicing of exon IIIb and to silence exon IIIc. However, although we show that hnRNP M does indeed play a role in exon IIIc silencing, we did not find evidence that it can increase exon IIIb inclusion using the assays described here. Thus, it is likely that other factors that also bind to ISE/ISS-3 may be involved in exon IIIb activation and that the coordinated activities of several factors together achieve these dual functions. A straightforward mechanism by which the ISE/ISS-3 element might coordinate these activities would be through the function of a factor whose expression was restricted to epithelial cell types. For this reason, we used extracts from a relevant cell type that expresses FGFR2-IIIb for these experiments to identify regulatory factors that bind this element. However, expression of hnRNP M was not noted to be significantly different between cells expressing FGFR2-IIIb or FGFR2-IIIC. Therefore, further identification and characterization of other factors that associate with the element will be necessary to clarify the mechanism by which cell-type-specific regulation is achieved.

Our experiments show that binding of hnRNP M to ISE/ISS-3 affects the efficiency of exon IIIc silencing as mutations or substitutions of the element to yield sequences that abrogate hnRNP M binding reduce the degree of exon IIIc skipping. However, it is also clear that exon IIIc silencing can occur in the absence of this element. Thus, binding of hnRNP M to this element alone only contributes a fraction of the protein’s total silencing activity on exon IIIc. The most likely mechanism by which hnRNP M plays a role in exon IIIc skipping is therefore likely to involve binding at multiple sites flanking or within exon IIIc. Such a requirement of multiple binding sites for splicing regulators to achieve maximal effects on splicing has been observed in many cases and appears to be a common theme in splicing regulation (1, 31). For example, the ability of other factors that have been shown to promote efficient exon skipping such as hnRNPA1 and polypyrimidine tract-binding protein (hnRNPI) involves binding at multiple sites on both sides of the regulated exon (8, 29, 32). Further characterization of other sites within the FGFR2 transcript that are also bound by hnRNP M should provide further insights into the mechanism by which it promotes exon IIIc skipping.

We also determined that hnRNP M can influence the splicing of several unrelated alternative exons. In the case of prepro-
tachykinin exon 4 and the α-exon of FGFR1 it promoted exon skipping as we observed for exon IIIc. Conversely, hnRNP M promoted inclusion of a troponin-derived exon flanked by muscle specific ISEs. Thus, unlike other well described hnRNP proteins, such as hnRNP A1 and polyuridyminate tract-binding protein, which have been shown to silence splicing of a broad array of alternative exons, the repertoire of functions of hnRNP M includes the ability to both positively and negatively influence exon definition (29, 32). In this regard, the function of hnRNP M is similar to the splicing regulators Nova-1, Fox-1, CUG, and Etr-like factors (CELFs), and hnRNP L, which have likewise been shown to promote exon inclusion or exclusion, depending on the context. For these splicing regulatory proteins, the position and proximity of their binding sites relative to regulated exons have been shown to determine which of these activities predominates (31, 33–36). Interestingly, members of the CELF family of splicing regulatory proteins were previously shown to activate exon R35 (28) inclusion through binding the muscle-specific ISE elements flanking the R35 (28) element (17). In fact, the ability of hnRNP M to activate exon R35 (28) inclusion was nearly equivalent to that achieved in a co-transfection with a plasmid expressing CELF 6 (data not shown). Thus, these results suggest that, in addition to the CELFs, hnRNP M may also contribute to the regulation of muscle-specific exons. In any case, the fact that hnRNP M affected the splicing of each minigene we tested suggests that, similar to other well characterized splicing factors, it may play a general role in alternative splicing of numerous transcripts.

The present manuscript is the first demonstration of which we are aware in which hnRNP M has been shown to regulate splicing in metazoans. Furthermore, this is the first report in which binding to a specific auxiliary cis-element has been shown to directly correlate with the ability of hnRNP M to regulate splicing in any organism. However, potential roles for hnRNP M in constitutive as well as regulated splicing have been suggested by several previous studies in diverse species. hnRNP M was originally identified as an abundant member of a core set of 24 polypeptides present in immunopurified hnRNP complexes (37). Among these proteins, hnRNP M was shown to associate with single-stranded DNA in a heparin-resistant manner, consistent with binding to RNA as a single-stranded nucleic acid-binding protein. Like other members of the core hnRNP family, hnRNP M is characterized by several copies of the classic RNA recognition motif that mediate RNA binding, as well as an unusual methionine and arginine rich motif, the MR repeat motif (25). The roles of each of the hnRNP proteins in splicing are not entirely clear for each of these abundant RNA-binding proteins. However, hnRNP M is among several that are not only present in the pre-spliceosomal H complexes that assemble in vitro, but also in mature spliceosomes. In fact several studies, including comprehensive proteomic analyses of in vitro purified spliceosomes have shown that hnRNP M is present not only in H complex, but stays associated with RNA transcripts through the stepwise progression of spliceosomal assembly from E/CC to A, B, and the relatively mature C complex (38–41). Further support for a role of hnRNP M in splicing was provided by studies showing that antibodies against the protein inhibited splicing in vitro (42). This study also noted that heat shock-induced inhibition of splicing was associated with a loss of hnRNP M from spliceosomal complexes, which may further implicate it in constitutive splicing.

The suggestion that hnRNP M may also play a role in splicing regulation was recently proposed based on studies of an insect hnRNP M orthologue, HRP59 (43). Immunoprecipitation of mRNAs associated with Drosophila melanogaster HRP59 revealed a number of mRNAs that contained an exonic GGAGG sequence motif, which resembled the previously described GAR type of exonic splicing enhancer (44). Although some in vitro binding preference for an RNA sequence containing GGAGG (compared with two control RNAs) was shown, no direct evidence was provided by this study that these sequences within the transcripts that contained them were indeed involved in splicing (43). Nonetheless, more recently these same investigators used RNA interference to show that D. melanogaster HRP59 can regulate splicing of its own transcript as well as that of another transcript by functioning as a negative regulator of splicing (45). Although these studies, together with our findings, suggest a conserved role of hnRNP M proteins in splicing regulation, several questions remain. Perhaps most important will be the identification of an optimal sequence motif that mediates sequence-specific binding of hnRNP M. Our results showing that binding of hnRNP M to GU-rich sequences in the intronic ISE/ISS-3 are consistent with previous reports showing a binding preference for poly(G) and poly(U) homopolymers (25, 46). This contrasts with the suggestion that GGAGG exonic sequences represent an optimal binding site for the insect orthologue. It may be that further studies may indeed demonstrate that HRP59 likewise binds to GU-rich sequence elements, including those present in introns. Alternatively, it may be that there has been divergence at the level of sequence binding specificity between these related hnRNP protein family members. Notably, the unique MR repeat motif in metazoan hnRNP M is not conserved in either the Chironomus tentans or D. melanogaster HRP59 protein, suggesting that these orthologues may indeed be functionally distinct. Further studies will also be needed to dissect the mechanisms whereby hnRNP M can affect splicing by either positively or negatively regulating exon inclusion in different transcripts. More detailed identification of additional binding sites for hnRNP M flanking FGFR2 exon IIIc as well as near other exons that it regulates should begin to clarify these questions.

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