Tissue-dependent isoforms of mammalian Fox-1 homologs are associated with tissue-specific splicing activities

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ABSTRACT

An intronic hexanucleotide UGCAUG has been shown to play a critical role in the regulation of tissue-specific alternative splicing of pre-mRNAs in a wide range of tissues. Vertebrate Fox-1 has been shown to bind to this element, in a highly sequence-specific manner, through its RNA recognition motif (RRM). In mammals, there are at least two Fox-1-related genes, ataxin-2 binding protein 1 (A2BP1)/Fox-1 and Fxh/Rbm9, which encode an identical RRM. Here, we demonstrate that both mouse Fxh and A2BP1 transcripts undergo tissue-specific alternative splicing, generating protein isoforms specific to brain and muscle. These tissue-specific isoforms are characterized for their abilities to regulate neural cell-specific alternative splicing of a cassette exon, N30, in the non-muscle myosin heavy chain II-B pre-mRNA, previously shown to be regulated through an intronic distal downstream enhancer (IDDE). All Fxh and A2BP1 isoforms with the RRM are capable of binding to the IDDE in vitro through the UGCAUG elements. Each isoform, however, shows quantitative differences in splicing activity and nuclear distribution in transfected cells. All Fxh isoforms and a brain isoform of A2BP1 show a predominant nuclear localization. Brain isoforms of both Fxh and A2BP1 promote N30 splicing much more efficiently than do the muscle-specific isoforms. Skeletal muscles express additional isoforms that lack a part of the RRM. These isoforms are incapable of activating neural cell-specific splicing and, moreover, can inhibit UGCAUG-dependent N30 splicing. These findings suggest that tissue-specific isoforms of Fxh and A2BP1 play an important role in determining tissue specificity of UGCAUG-mediated alternative splicing.

INTRODUCTION

Alternative splicing of pre-mRNA is one of the fundamental mechanisms for the regulation of gene expression in higher eukaryotes (1,2). Developmentally regulated, cell type- or tissue-specific, and signal-induced alternative splicing of pre-mRNAs takes place in multicellular organisms throughout their lifetimes. Misregulation or abnormalities in pre-mRNA splicing, in some instances, leads to cellular dysfunctions found in human and animal diseases (3–5). Using various model systems of regulated alternative splicing, a number of pre-mRNA features that influence alternative splice site selection have been defined (1,2). These include enhancer and repressor RNA sequences located in exons and introns. Identification of RNA-binding proteins targeting these cis-regulatory elements is currently in progress. In vertebrates, participation of the SR family proteins and hnRNP proteins, such as PTB and hnRNP A1, in alternative splicing regulation via binding to the cis-regulatory elements have been shown in many tissue-specific splicing models (6,7). Although these RNA-binding proteins are ubiquitously expressed, their different abundance in different cells, differences in their post-translational modifications in different cellular contexts and their different abilities to assemble multiprotein complexes in different pre-mRNA contexts are thought to contribute to the determination of cell type-specific patterns of alternative splicing. For the last few years, tissue-specific and tissue-enriched RNA-binding proteins have begun to be identified as splicing regulators. These include brain-specific (or enriched) Nova-1, nPTB (brPTB) and some of the CELF family proteins (8–12). Discovery of these proteins has had a great impact on studies aimed at understanding the molecular mechanisms of alternative splicing regulation.
One of the intronic cis-elements, which are involved in tissue-specific or differentiation stage-dependent regulation of alternative splicing, is the hexanucleotide UGCAUG. The importance of this element was originally recognized in fibronectin pre-mRNA by Huh and Hynes (13). Since then, alternative splicing specific to a variety of tissues or cell types, including neural cells, muscles, epithelial cells and erythrocytes, has been shown to be modulated via this element (14–21). Recently, Jin et al. (21) discovered that a zebrafish homolog of Caenorhabditis elegans Fox-1 could bind specifically to the pentanucleotide GCAUG by in vitro selection from randomized RNA sequences. This pentanucleotide is almost identical to the hexanucleotide UGCAUG except for the first U. Moreover, the zebrafish Fox-1 homolog, as well as the mouse Fox-1 homolog, are capable of repressing the inclusion of an alternative cassette exon of the ATP synthase F1γ pre-mRNA via binding to GCAUG, which mimics muscle-specific exclusion of this exon. This mouse homolog is identical to the ataxin-2 binding protein 1 (A2BP1), which has been previously cloned in humans and mice as the cDNA encoding a protein, which interacts with ataxin-2, the product of the causative gene for spinocerebellar ataxia type 2 (23,24). In addition to A2BP1/Fox-1, another mouse homolog of C elegans Fox-1, Fxh, has been independently cloned as a cDNA, which is induced by androgen in motor neurons (25). Of note is that A2BP1/Fox-1 and Fxh share an identical RNA recognition motif (RRM) at the amino acid level. Therefore, two genes in the mouse genome encode homologs of nematode Fox-1. According to the names given by the authors of the respective cloning, we used the nomenclature of A2BP1 and Fxh in this report. A2BP1 and Fxh have been named A2BP1 and Rbm9, respectively, in the human and mouse genomes.

We have been studying regulatory mechanisms of neural cell-specific alternative splicing using the non-muscle myosin heavy chain II-B (NMHC-B) gene as a model system (14,26). NMHC-B mRNA is expressed ubiquitously. However, an alternative exon, N30, which encodes a 30 nt coding sequence, is included in the mRNAs from some neural cells, but is skipped in those from all other cells in mammals and birds (27,28). In cultured cells, a switch in N30 splicing from exclusion to inclusion can be seen in neural retinoblastoma Y79 cells during the post-mitotic and differentiated stages triggered by butyrate treatment. We have previously defined an intronic distal downstream enhancer element (IDDE), which confers nuclear cell specificity on N30 inclusion, using this cell line (14). The IDDE includes two copies of UGCAUG. Mutation of these hexanucleotides results in N30 skipping in post-mitotic differentiated Y79 cells.

In this study, we investigated the possible involvement of A2BP1 and Fxh in the regulation of N30 splicing. To this end, we have isolated cDNA clones for A2BP1 and Fxh from brain and muscles. cDNA cloning revealed the existence of tissue-specific (enriched) isoforms of both A2BP1 and Fxh. Of importance, different isoforms of A2BP1 and Fxh show different activities with respect to N30 splicing as well as different subcellular localizations.

**MATERIALS AND METHODS**

**Database disposition**

The sequences reported in this paper have been deposited in the GenBank database with accession numbers AY659951 (F011), AY659952 (F411), AY659953 (F402), AY659954 (A016), AY659955 (A030), AY659956 (A713), AY659957 (A715) and AY659958 (A704).

**RNA preparation and RT–PCR**

Total RNAs were isolated from mouse tissues and cultured cells using an RNA isolation kit (Stratagene). To obtain the full-length coding regions of cDNAs for Fxh and A2BP1, RT–PCRs were performed using Superscript II RNase H– reverse transcriptase (Invitrogen) and Pfu Turbo DNA polymerase (Stratagene). The PCR primers used to obtain all Fxh cDNAs were 5’-ctcaggcctcactagatfag-gaaaagaaaatagtgtaactc-3’ and 5’-ctcaggcctcctagaaataagggggcaattcgcgtga-3’. The upstream primers for the brain (A016 and A030) and muscle (A713, A715 and A704) A2BP1 cDNAs were 5’-ctcaggcctcactataaatgtaagagaccgactt-3’ and 5’-ctcaggcctcactagctgataggtggaggtttc-3’, and the downstream primer for all A2BP1 cDNAs was 5’-ctcaggcctcctagagatagggagacacggtgtgtaccc-3’. Lower case letters represent adapter sequences including restriction enzyme sites. 5’ Rapid amplification of cDNA ends (RACE) was performed using Marathon-Ready cDNA (BD Biosciences Clontech). For the analysis of minigene and NMHC-B mRNAs, RT–PCRs were performed as described previously (14,26). Sequences of primers P1–P9 shown in Figures 1, 4 and 6 are as follows: P1, 5’-aattcaccagcaaccagat-3’; P2, 5’-tagagggatataagagtgt-3’; P3, 5’-cagaggccgcaagtgtagtttgatgcc-3’; P4, 5’-gagaggccggcaggggcag-3’; P5, 5’-ccgtgagtgcgaccaccttcacac-3’; P6, 5’-caagcgccagttggcagggtg-3’; P7, 5’-agagaagaaaaagacataaatatacc-3’; P8, 5’-ctcctcaccacccagcctcctgtgg-3’; and P9, 5’-ccggtagttattaaaaatccttcaag-3’.

**Preparation of expression constructs and minigenes**

The cDNAs of Fxh and A2BP1 were introduced into a plasmid pCS3 + MT, which contains a myc-epitope, and its modified version pCS3 + MT + NLS, which in addition contains the nuclear localization signal (NLS) of the SV40 large T antigen (26). Minigenes G, J without the IDDE, and H with the wild-type IDDE are the same as minigenes W, D4 and Cm0, respectively, in ref. (14). The 201 nt IDDEs with mutations in vitro Template DNAs for minigenes were prepared by PCR using the wild-type and mutant IDDEs in the minigenes as templates and an upstream primer that included the GTGGAAGAAAGGACCATAATAT-leucine TGCATG sequences at the 5’ side.

**Electrophoresis mobility shift assay**

Template DNAs for in vitro RNA transcription were prepared by PCR using the wild-type and mutant IDDEs in the minigenes as templates and an upstream primer that included the T7 promoter sequence at the 5’ end. The probe and competitor RNAs were transcribed by T7 RNA polymerase in the presence of [α-32P]UTP and a trace amount of [35S]UTPoxS, respectively, using a MAXIscript kit (Ambion). Mole concentrations of synthesized RNAs were estimated by radioactivity. Fxh and A2BP1 proteins with a myc tag were synthesized in vitro by using a TNT quick-coupled
transcription/translation system (Promega) from pCS3 + MT constructs, which include the SP6 promoter. Binding reactions were carried out in a 10 μl mixture that contains 10 mM HEPES (pH 7.9), 2 mM MgCl₂, 50 mM KCl, 5% glycerol, 0.5 mM DTT, 5 μg tRNA, 1 μl reticulocyte lysate reaction mixture and 15 fmol of probe, on ice for 20–30 min. An aliquot of 5 μg of heparin was added to the reaction 10 min before gel electrophoresis. The reaction mixtures were analyzed by electrophoresis in a 6% polyacrylamide gel using a 0.5 · TBE buffer (Invitrogen).

**Cell culture and transfection**

The human retinoblastoma cell line Y79 was cultured and transfected with DNAs as described previously (14,26). Total amounts of transfected DNAs were adjusted to be constant by addition of the empty vector. Either Lipofectin (Invitrogen) or Effectene transfection reagent (Qiagen) was used for the transfection. For stable transfection, the pCS3+MT expression constructs were co-transfected with a plasmid carrying a neomycin resistant gene and selected by 0.2 mM geneticin (Invitrogen). For differentiation of Y79 cells, cells were plated on the poly-n-lysine-coated plates and then treated with 2.0–2.5 mM sodium butyrate for 4–5 days. HeLa cells were cultured as described and transfected with DNA using Effectene reagent (14,26).

**Immunoblot analysis**

Samples that required both protein and mRNA analysis were split upon harvesting. Total cell proteins were subjected to SDS–PAGE and blotted as described previously (26). The primary antibodies used were monoclonal antibodies to a myc-epitope (Invitrogen) and green fluorescent protein (GFP) (Clontech). Binding of antibodies was detected with the SuperSignal System (Pierce) or ECL (Amersham).

**Immunofluorescent microscopy**

HeLa or Y79 cells grown in a four-chamber glass slide were transfected as described above. Cells were fixed with 10% formaldehyde 24–48 h after transfection, and permeabilized with 0.5% Triton X-100 then blocked with 5% goat serum.
Primary antibodies used were mouse anti-myc (Invitrogen), rabbit anti-NMHC-B (29). Secondary antibodies were Alexa Fluor 594 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). DAPI was used for DNA staining. Specimens were mounted in ProLong antifade kit (Molecular Probe). The images were collected using Leica SP confocal microscopy (Leica).

RESULTS

Tissue-dependent isoforms of Fxh and A2BP1 and their subcellular distribution

The A2BP1 mRNAs are detected almost exclusively in brain and striated muscles (heart and skeletal muscles) in adult mice, whereas the Fxh mRNAs are expressed in a wide variety of tissues with the highest expression in brain and heart ([21,25] and S. Kawamoto, unpublished data). These expression profiles prompted us to characterize the mRNAs of A2BP1 and Fxh in brain and muscles. We have cloned cDNAs for two gene transcripts from brain, heart and skeletal muscles by RT–PCR and 5′ RACE. The isolated cDNAs are schematically presented with genomic structures in Figure 1 (for amino acid sequences see also Figure 8). Both gene transcripts are found to undergo tissue-specific alternative splicing. In both cases, brain and striated muscles express unique splice variants generated by mutually exclusive splicing of exons B40 and M43, respectively, which provide different coding sequences in the middle of the carboxyl half of the molecules. Southern blot analysis of the RT–PCR products of the Fxh mRNAs from the different tissues probed by oligonucleotides corresponding to B40 and M43 shows that M43 is exclusively used in heart and skeletal muscles, while B40 is predominantly used in brain (Figure 2A). Digestion of the RT–PCR products of the A2BP1 mRNAs with restriction enzymes unique to B40 and M43 demonstrates that B40 and M43 are used almost exclusively in brain and muscles, respectively (Figure 2B).

A2BP1 contains an additional cassette type alternative exon A53, consisting of 53 nt. Inclusion and exclusion of exon A53 results in two different amino acid sequences at the C-terminal region owing to a frame shift. In the case of the A2BP1 gene, moreover, it appears that brain and striated muscle utilize alternative promoters, resulting in different amino acid sequences at the N-terminus. The 5′ RACE of skeletal muscle mRNAs yielded essentially a single species of sequence with 29 unique N-terminal amino acids. The 5′ RACE using brain mRNAs; however, yielded multiple products with multiple deduced amino acid sequences, all of which differ from the muscle amino acid sequence at the very N-terminus. Here, we focus our analysis on a clone containing nine unique amino acids at the N-terminus, which was obtained most frequently. Exon–intron organization of Fxh and A2BP1 shows remarkable similarities and a RRM is encoded in four exons (Figure 1B). Of note is that the significant amounts of Fxh and A2BP1 mRNAs from skeletal muscles are missing a part of the RRM by exon skipping. Typically, as shown in Figure 2C, they lack the 93 nt exon that encodes RNP1, one of the two most critical motifs of the RRM (30). Some of them lack almost the entire RRM (e.g. F402 in Figure 1A).

To examine the subcellular distribution of each isoform of Fxh and A2BP1, myc-tagged proteins were transiently expressed in cultured cells and immunostained with an anti-myc antibody. Initially, HeLa cells were used to investigate subcellular localization, since these cells are more suited for these studies. Representative confocal images are shown in Figure 3A. DAPI and anti-NMHC-B antibodies serve as markers for nuclei and cytoplasm, respectively. As noted, the ratio of protein distributed between nuclei and cytoplasm differs among the proteins. All isoforms of Fxh have a predominant nuclear localization. The brain isoform of A2BP1 without the A53 exon (A016) localizes to both nuclei and cytoplasm, whereas the other brain isoform with the A53 exon (A030) localizes predominantly to cytoplasm with only a minimum being in the nuclei. The relative amounts of both muscle isoforms of A2BP1 (A713 and A715) are somewhat between the amounts of the two brain isoforms. These data are summarized in Figure 1A. The subcellular distribution of representative isoforms (F011, A016 and A030) was also examined in retinoblastoma Y79 cells, which were used as host cells for the transfection experiments in order to characterize splicing activities of Fxh and A2BP proteins (see below). Although Y79 cells have a spherical shape and have only thin cytoplasm,
exogenously expressed isoforms of Fxh and A2BP1 show a similar subcellular distribution as in HeLa cells (Figure 3B). F011 localizes predominantly to nuclei, A016 to both nuclei and cytoplasm and A030 predominantly to cytoplasm.

Specific interaction of Fxh and A2BP1 with IDDE via a hexanucleotide UGCAUG

Fxh and A2BP1 share an identical RRM and A2BP1 has been reported to bind specifically to the pentanucleotide GCAUG (21). We have previously reported that the IDDE of the NMHC-B transcript, which is indispensable for the regulation of neural cell-specific cassette type exon N30 splicing, has two copies of GCAUG (14). Therefore, we investigated whether Fxh and/or A2BP1 bound to the IDDE. Electrophoretic mobility shift assays (EMSAs) were carried out using labeled IDDE and in vitro transcribed and translated Fxh and A2BP1, which include a myc-epitope. Since all Fxh and A2BP1 isoforms, except F402 and A704, contain the identical RRM, representative isoforms were analyzed. The expression of F011 in reticulocyte lysate causes the formation of a RNA–protein complex whose migration shift distinguishes it from those of the control reticulocyte lysate (C in Figure 4B, lanes 3 and 10). The unlabeled wild-type IDDE competes with the probe efficiently for the formation of the specific complex C (Figure 4B, lane 4). On the other hand, the mutant mc (Figure 4A), which has a mutation in both copies of UGCAUG, does not (Figure 4B, lane 7). The mutant ma, which has a mutation in the hexanucleotide at the 5' side, shows less efficient competition, compared with mb, which has a mutation in the 3' side of the hexanucleotide (Figure 4B, lanes 5 and 6), indicating that the nucleotides at the 5' side are more important than those at the 3' side. The presence of an anti-myc antibody, but not a non-specific antibody, inhibits the formation of the specific complex (Figure 4B, lane 8). Synthesis of the full-length F011 protein using a reticulocyte lysate and specificity of the myc antibody for the expressed protein are verified by immunoblot analysis (Figure 4B, lanes 11 and 12). A016 and A030 show essentially identical results to those with F011 (data not shown). These results indicate that Fxh and A2BP1 can bind to the IDDE and that the hexanucleotide UGCAUG is required for their binding.

Fxh and A2BP1 enhance N30 inclusion in a UGCAUG-dependent manner

To study whether Fxh and A2BP1 regulate neural cell-specific splicing via binding to UGCAUG, the Fxh and A2BP1 expression constructs were co-transfected into retinoblastoma Y79 cells with a number of the reporter minigene constructs, which include the wild-type or a mutant version of UGCAUG in the IDDE (Figure 4A). Minigenes H and J consist of the exons E5, N30 and E6 and their flanking introns with some deletions in the introns. The IDDE with or without mutations in the...
hexanucleotide is included or excluded between N30 and E6. As described above, each isoform of Fxh and A2BP1 enters the nucleus to a different extent. To see the effects of different proteins on N30 splicing itself, independent of their different properties in nuclear localization, an exogenous NLS was added to the expressed proteins. Since Fxh and A2BP1 proteins contain the identical RRM, and F011, A016 and A030 all show the same UGCAUG-dependent binding to the IDDE in vitro, F011 and A030 were used for these experiments. Host cells Y79 at the proliferating stage exclude the N30 exon in ~90% of the endogenous NMHC-B mRNAs (e.g. see Figure 6A, lane 1).

As shown in Figure 4C, the mRNAs derived from minigene J exclude N30 without exogenous expression of Fxh or A2BP1 in Y79 cells, similar to the endogenous NMHC-B mRNAs (Figure 4C, upper panel, lanes 1–5). However, in the presence of exogenous expression of F011 and A030, the N30 inclusion is increased (Figure 4C, upper panel, lanes 7 and 12). The N30 inclusion is absolutely dependent on the presence of the IDDE (Figure 4C, upper panel, lanes 6 and 11). Moreover, mutation
of either one of the two copies of UGCAUG (ma, mb) abolishes the inclusion of N30 (Figure 4C, upper panel, lanes 8–10, 13–15). In the context of minigene H, which contains shorter introns, the larger extent of N30 inclusion is induced by either F011 or A030 overexpression (Figure 4C, middle panel, lanes 7 and 12). The N30 inclusion of the minigene H mRNAs also depends on the presence of the IDDE (Figure 4C, middle panel, lanes 6 and 11). Mutation of both copies of UGCAUG (mc) results in a complete loss of N30 inclusion (Figure 4C, middle panel, lanes 6 and 11). The mutant ma shows stronger inhibition of N30 inclusion compared with mb (Figure 4C, middle panel, lanes 8 and 9). This observation is consistent with the competition experiments of the EMSA shown in Figure 4B, indicating that the 5′ hexanucleotide is more important than the 3′ hexanucleotide for binding of Fxh or A2BP1 to the IDDE as well as an activation of N30 splicing. Comparable amounts of F011 and A030 are expressed in each transfection as verified by immunoblots using an anti-myc antibody (Figure 4C, lower panel, lanes 6–17). Since minigenes J and H lack a portion of the intron between exons N30 and E6 and, therefore, the IDDE is located ~100 nt downstream of N30, instead of 1.5 kb as in the native gene, we also analyzed the effects of Fxh and A2BP1 on the N30 splicing of the wild-type minigene G, which includes full-length introns among E5, N30 and E6 (Figure 4A). As shown in Figure 4C (right panel), both F011 and A030 are capable of promoting N30 inclusion, with F011 showing a higher activity (Figure 4C, lanes 16 and 17). Although A030 can promote N30 inclusion as efficiently as F011 in the contexts of the minigene J and H transcripts, it can do so less efficiently than F011 in the context of the minigene G transcript. Interpretation of this observation will be discussed below.

Taken together, Fxh and A2BP1 can activate N30 inclusion in an IDDE-dependent manner. The hexanucleotide motif UGCAUG is indispensable for this activation.

Differential activities of alternatively spliced isoforms of Fxh and A2BP1 in promoting N30 inclusion

Both Fxh and A2BP1 mRNAs are expressed in brain and A2BP1 is also expressed in striated muscles and Fxh is expressed in an even wider variety of tissues. As demonstrated above, however, both Fxh and A2BP1 transcripts undergo tissue-dependent alternative splicing, producing muscle-specific and brain-enriched isoforms. Therefore, the relative activity of individual isoform of Fxh and A2BP1 in promoting N30 inclusion was compared using minigene G.

First, in order to evaluate the relative specific activity of each isoform in the splicing reaction separately from its ability to localize to nuclei, an exogenous NLS was included in the expressed protein to equalize the nuclear concentration of the expressed protein in these experiments. Essentially, all of the expressed proteins with the exogenous NLS are localized to the nucleus (data not shown). Therefore, the relative nuclear concentrations of the expressed proteins can be estimated easily by immunoblots. As shown in immunoblots in Figure 5A, similar quantities of proteins are expressed in a dose-dependent manner. Expression of the brain isoform F011 causes a dose-dependent increase in N30 inclusion and the extent of N30 inclusion reaches a plateau with ~85% of the mRNAs including N30. In contrast, inclusion of N30 promoted by F411, the predominant isoform from skeletal muscles, reaches a plateau with only 40% of the mRNAs. With respect to A2BP1, the brain isoform A030 shows higher activity in N30 inclusion than the muscle isoform A715, which shows almost no activation, as shown in Figure 5A. Including additional isoforms, the activities of individual isoforms with an exogenous NLS in promoting N30 inclusion are shown in Figure 5B (lanes 8–13) and are also summarized in Figure 1A (nls). Special care was taken to ensure that a similar quantity of each protein was expressed and, if not, different amounts were tested to obtain comparable expression. In the presence of the exogenous NLS (nls), F011 and A016 show the highest activities among all isoforms tested. A30 shows a considerably lower activity than A016. Each of the muscle isoforms (F411, A713 and A715) has a lower activity than that of their brain counterparts (F011, A016 and A030, respectively). As expected, isoforms lacking a part or all of the RRM (F402 and A704) have no activity for N30 inclusion.
Next, the splicing activities of the wild-type proteins without an exogenous NLS were examined. Representative data are shown in Figure 5B (lanes 2–7) and the relative activities are summarized in Figure 1A (wt). The protein amounts detected by immunoblots in these experiments represent the total amounts of the proteins distributed to both the nuclei and the cytoplasm. The splicing activities of the wild-type proteins are consistent with the activities that combine the splicing activities of the proteins with the exogenous NLS and the activities of the native proteins to localize to nuclei. In the absence of the exogenous NLS (wt), the brain isoforms F011, and A016 to a lesser extent, are still capable of activating N30 inclusion efficiently. The muscle isoforms for both Fxh and A2BP1 (F411, A713 and A715), as well as the brain isoform A030, show only minimal activities. Therefore, F011 and A016 appear to have the most physiological relevance to N30 splicing activation.

Overexpression of Fxh and A2BP1 activates N30 inclusion of endogenous NMHC-B mRNAs

The human NMHC-B gene consists of 41 constitutive exons and 3 alternative exons. Its pre-mRNA is ~156 kb in length and it is much more complex than the pre-mRNA from the minigenes. In addition, the minigene pre-mRNAs are driven by a heterologous promoter. Therefore, we next examined if Fxh and A2BP1 were capable of promoting N30 inclusion of the endogenous transcript. Y79 cells were stably transfected with the expression construct for F011 or A016. Both F011 and A016 are enriched in the brain and show higher activation of N30 inclusion in the minigene transcripts. mRNAs encoding endogenous NMHC-B were analyzed by using RT–PCR. As shown in Figure 6, the inclusion of exon N30 with and without another alternative exon, R18, in the endogenous mRNAs is markedly increased in the clones which were stably transfected with the construct for F011 or A016 containing an exogenous NLS (Figure 6, lanes 4 and 5). Although to a lesser extent, transfection of the wild-type constructs without an exogenous NLS also results in a significant increase in N30 inclusion (Figure 6, lanes 2 and 3). Thus, exogenously expressed Fxh and A2BP1 are capable of activating N30 inclusion not only in the transcripts from the minigenes, but also in those from the native NMHC-B gene in Y79 cells.

Fxh may cooperate with other factor(s) to promote N30 inclusion

To address the role of endogenous Fxh and its potential interaction with other proteins in promoting N30 inclusion, we made use of an isoform of Fxh, F402, which lacks the RRM. The mutant proteins lacking an RRM for other RNA-binding proteins have previously been reported to function in a dominant-negative fashion and inhibit the activities of the wild-type proteins (26,31). Therefore, the effects of F402 on the N30 inclusion of minigene H mRNAs were examined in the context of the Y79 cells treated with butyrate. Upon butyrate treatment, as reported previously, Y79 cells enter in a post-mitotic and differentiated stage, and importantly, the endogenous as well as the minigene mRNAs in those cells include N30 to a large extent, unlike those in the untreated cells that predominantly exclude N30 (14). As shown in Figure 7, in the absence of exogenous expression of F402, large quantities of the mRNAs derived from minigene H-wt, which contains the wild-type IDDE, include N30 (Figure 7, upper panel, lane 4). In contrast, only small quantities of N30 inclusion are detected in the mRNAs from minigene H-mc, which has mutations in both copies of UGCAUG in the IDDE (Figure 7, upper panel, lane 8). Therefore, the butyrate-treated Y79 cells contain factor(s), which are capable of activating the UGCAUG-dependent N30 inclusion. Co-transfection of the wild-type minigene H-wt with the F402 expression construct causes a dose-dependent inhibition of N30 inclusion (Figure 7, lanes 1–3), indicating that F402 has an antagonistic effect on the endogenous factors with respect to N30 inclusion. Notably, the UGCAUG-independent N30 inclusion seen in the mutant minigene H-mc is not affected by the co-expression of F402 (Figure 7, lanes 5–7), indicating that the inhibitory activity of F402 depends on the UGCAUG element. The parallel experiment with the F011 expression construct does not show a significant effect on N30 splicing in either the wild-type or mutant minigene (Figure 7, lanes 9–16). This may be due to the fact that N30 inclusion of the wild-type minigene mRNAs has already reached a maximal level and is consistent with the idea that butyrate-treated Y79 cells contain sufficient amounts of protein(s) functionally equivalent to F011, which can promote N30 inclusion in a UGCAUG-dependent manner. Since F402 does not bind to the UGCAUG element, and has a UGCAUG-dependent antagonistic effect on N30 splicing, it most probably disrupts protein–protein interactions of the endogenous proteins that are required for the UGCAUG-dependent activation of N30 splicing. Endogenous Fxh (and/or A2BP1) would be a good candidate whose function could be antagonized by exogenously expressed F402. Thus, this observation suggests that the endogenous Fxh has an effect on the activation of N30 splicing and that other proteins cooperate with Fxh for N30 activation. Since muscle cells express the RRM-defective isoforms to a significant extent (Figure 2C) and the wild-type F402 localize to...
Subcellular compartments at steady-state differ among the isoforms. In agreement with Jin et al. (21), substantial amounts of the brain isoform A016 are detected in nuclei. Other A2BP1 isoforms, the brain isoform A030 and the muscle isoforms A713 and A715, are only poorly detected in nuclei. This observation is consistent with the reports where endogenous A2BP1 in cerebellar Purkinje cells, hippocampus neurons and cardiac myocytes were shown to be localized essentially to the cytoplasm (23,24). Thus, inclusion and exclusion of A53 and differences in the very N-terminal sequences results in A2BP1 isoforms with a distinct subcellular localization. It is likely that A2BP1 proteins have multiple roles, involving both nuclear and cytoplasmic events. In contrast, all three Fxh isoforms predominantly localized to the nuclei. Therefore, in terms of their localization, Fxh proteins are better candidates for regulators of the pre-mRNA splicing that takes place in nuclei. Of note, however, our preliminary results of 5’ RACE, as well as the EST database, detect multiple 5’ end sequences for both Fxh and A2BP1 mRNAs, which are presumably generated by alternative promoters and alternative splicing. The diversity of the 5’ end cDNA sequences leads to the generation of a number of unique N-terminal amino acid sequences. Therefore, this study does not exclude the possible existence of other isoforms with different subcellular localizations for both Fxh and A2BP1. Our study also does not exclude the possibility that some of the isoforms translocate between the nucleus and the cytoplasm following stimuli.

The main aim of this study is to determine the relative activities of tissue-dependent isoforms of Fxh and A2BP1 in neural cell-specific and UGCAUG element-dependent alternative splicing. To obtain an indication of the relative specific activity of each isoform in transfected cells, the same amounts of the expressed proteins should be available for the splicing reaction in the nuclei. For this reason, an exogenous NLS was included in the expressed proteins. Essentially, all of the expressed proteins with the exogenous NLS localized to nuclei. Thus, the amounts of the expressed proteins determined by immunoblots represent the nuclear concentrations. The analysis using the proteins expressed with the exogenous NLS allowed us to compare directly the splicing activities of these proteins. Furthermore, this analysis also allowed us to define the critical regions of the proteins for splicing activation.

As shown in Figure 5, the splicing activities of the various isoforms of Fxh and A2BP1 are intrinsically different, regardless of the subcellular localization properties of the wild-type proteins. Among the isoforms tested in this study, F011 and A016, which include B40, are found to have higher activities in promoting N30 inclusion. When the primary amino acid sequences, outside of the RRM, of these two proteins are compared, the C-terminal regions (amino acids 190–377 of F011) show a higher homology with 71% identity, whereas the N-terminal regions (amino acids 1–112 of F011) show only 53% identity. The C-terminal region includes four subregions of nearly identical stretches of amino acids (Figure 8, I–IV). One subregion (II) includes 13 amino acids encoded by exon B40. Substitution of this subregion with exon M43 in Fxh causes substantial changes in amino acid sequences resulting in only a 21% identity in this region between F011 and F411. Another subregion (IV) is located at the C-terminal end and A030 lacks this homologous region by the inclusion of exon

**DISCUSSION**

Two major findings are described in this report. First, Fxh and A2BP1 facilitate neural cell-specific inclusion of the cassette-type exon via binding to the specific intronic sequence UGCAUG. In addition to a minigene model system, Fxh and A2BP1 are capable of facilitating N30 inclusion of the endogenous pre-mRNA. This result provides an important demonstration of physiological relevance and supports the notion that the NMHC-B pre-mRNA is likely to be the true target for Fxh or A2BP1-mediated regulation. However, whether the endogenous Fxh or A2BP1 regulates endogenous NMHC-B pre-mRNA splicing needs to be determined in a future study. In vertebrates, small interfering RNAs and gene targeting strategies have recently been used successfully to address the roles of endogenous splicing regulators in alternative splicing of endogenous target pre-mRNAs (8,32–36). A second and more novel finding is the identification of tissue-specific isoforms of Fxh and A2BP1 with different splicing activities as well as different subcellular localizations. This finding raises the possibility that the products of the Fxh and A2BP1 genes can contribute to a mechanism as to how tissue specificity of alternative splicing is achieved.

Many splicing factors are detected not only in the nuclei, but also in the cytoplasm (37). They are shuttling between the nucleus and the cytoplasm and, in some instances, extracellular stimuli trigger changes in subcellular distribution of these proteins. Such translocations have been reported for hnRNP A1 and PTB (38,39). Moreover, a number of RNA-binding proteins have been demonstrated to play a role in multiple steps during gene expression in different subcellular compartments, such as pre-mRNA processing in nuclei, mRNA export from nuclei to cytoplasm and mRNA localization, stability and translation in cytoplasm (37,40). Therefore, not surprisingly, Fxh and A2BP1 isoforms were found to be distributed in both the nuclei and the cytoplasm in HeLa and Y79 cells. However, the relative ratios of proteins distributed between the two

**Figure 7.** Antagonistic effect of the RRM-lacking isoform of Fxh on UGCAUG-dependent N30 inclusion in butyrate-treated Y79 cells. The indicated minigenes (see Figure 4A for diagram) were co-transfected with increasing amounts (0.7, 2 and 6 μg, indicated by right triangles) of the expression construct for the indicated protein or with an empty vector in Y79 cells. The cells were treated with butyrate to induce differentiation of Y79 cells. The minigene mRNAs were analyzed by RT–PCR. Dose-dependent protein expression was verified by immunoblot analysis using an anti-myc antibody.
A53, which results in a frame shift. Since F411 and A030 show poor splicing activation compared with F011 and A016, respectively, these two subregions of F011 and A016 appear to serve as activation domains, presumably by interacting with other proteins. This notion is supported by the finding that the RRM-lacking isoform F402, which includes the same subregions II and IV as F011, functions apparently as a dominant-negative mutant to the wild-type Fxh, consistent with the interpretation that the mutant and wild-type are competing to interact with other protein(s). To date, Fyn tyrosine kinase and estrogen receptor-α have been reported to interact with Fxh, and ataxin-2 with A2BP1 (23,41,42). Whether these

![Amino acid sequence alignments of Fxh and A2BP1 isoforms. Shaded and striped boxes indicate identical and similar amino acids, respectively, to F011. RRM and the amino acids encoded by exons B40 and M43 (B40/M43) as well as the highly homologous regions between F011 and A016 (I–IV) are indicated.](https://academic.oup.com/nar/article-abstract/33/7/2078/2401254)
proteins participate in the regulation of pre-mRNA splicing is currently unknown. Of interest, A030 with the exogenous NLS activates N30 splicing as efficiently as F011 in the pre-mRNAs derived from minigenes J and H, which contain the shorter intron, whereas this isoform poorly activates N30 splicing in the pre-mRNA from minigene G, which contains the full-length intron. This observation implies that the interactions of A030 with different factors are required in the different pre-mRNA contexts. Therefore, the isoforms of Fxh and A2BP1 described here may have different effects on other UGCAUG-regulated alternative splicing.

The involvement of the hexanucleotide UGCAUG in regulated alternative splicing has been experimentally demonstrated in a number of neural cell-specific, as well as other tissue-specific, model systems (13–21). This hexanucleotide element plays a role, in most cases, as an enhancer in regulating alternative splicing of cassette-type exons as well as mutually exclusive exons. Furthermore, computational analysis has revealed that UGCAUG is over-represented in the introns in which splicing is regulated, compared with the constitutively spliced introns (43). This analysis also pointed to the UGCAUG element as playing a role in the regulation of tissue-specific alternative splicing in a wide range of tissues, but not in specific tissues.

To date, KH-type splicing regulatory protein (KSRP) (44), A2BP1 (21) and Fxh (this study) are known to be capable of binding to UGCAUG. KSRP is expressed ubiquitously and tissue-specific variants of this gene have not been described so far. In this study, we have described the existence of tissue-dependent isoforms of Fxh and A2BP1, which, while not identical in some areas of the molecule, may contain the same RRM. The physiological relevance of these isoforms is that they have different splicing activities and different subcellular localizations. The brain isoforms promote N30 inclusion more efficiently than the muscle isoforms of both Fxh and A2BP1. The isoforms lacking the RRM are normally expressed to a significant extent in skeletal muscles. This isoform is incapable of activating N30 splicing and, moreover, can inhibit N30 inclusion. The properties of these isoforms are consistent with Fxh and A2BP1 acting as regulators for N30 splicing, since N30 is included in neuronal cells, but excluded in muscles. Therefore, despite the tissue-independent occurrence of UGCAUG as a regulatory element, given the tissue-dependent isoforms of the UGCAUG-binding proteins (Fxh and A2BP1) with different activities, the hexanucleotide UGCAUG could confer tissue specificity on regulated splicing. One of the major problems in understanding the mechanisms responsible for alternative pre-mRNA splicing is the manner in which tissue specificity is determined. In vertebrates, to date, only a few tissue-specific proteins have been identified as splicing regulators (8–12).

Here, we have shown that the tissue-dependent isoforms of the sequence-specific RNA-binding proteins, which themselves are generated by alternative splicing, have different activities in tissue-specific alternative splicing of target pre-mRNA. Therefore, these isoforms play a role in the determination of tissue specificity of target pre-mRNA splicing. The discovery of these tissue-dependent isoforms of the UGCAUG-binding proteins with different splicing activities adds an important new dimension to the molecular mechanisms responsible for regulating tissue-dependent alternative splicing mediated via UGCAUG.

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splicing event at 4.1R pre-mRNA during erythroid differentiation. 


