Fox-3 and PSF interact to activate neural cell-specific alternative splicing

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ABSTRACT

Fox-1 family (Fox) proteins, which consist of Fox-1 (A2BP1), Fox-2 (Rbm9) and Fox-3 (NeuN) in mammals, bind to the RNA element UGCAUG and regulate alternative pre-mRNA splicing. However the mechanisms for Fox-regulated splicing are largely unknown. We analyzed the expression pattern of the three Fox proteins as well as neural cell-specific alternative splicing of a cassette exon N30 of nonmuscle myosin heavy chain (NMHC) II-B in the mouse central nervous system. Histological and biochemical analyses following fluorescence-activated cell sorting demonstrate a positive correlation of N30 inclusion and Fox-3 expression. Further, we identified polypyrimidine tract binding protein-associated splicing factor (PSF) as an interacting protein with Fox-3 by affinity-chromatography. In cultured cells, enhancement of N30 inclusion by Fox-3 depends on the presence of PSF. PSF enhances N30 inclusion in a UGCAUG-dependent manner, although it does not bind directly to this element. Fox-3 is recruited to the UGCAUG element downstream of N30 in the endogenous NMHC II-B transcript in a PSF-dependent manner. This study is the first to identify PSF as a coactivator of Fox proteins and provides evidence that the Fox-3 and PSF interaction is an integral part of the mechanism by which Fox proteins regulate activation of alternative exons via a downstream intronic enhancer.

INTRODUCTION

Alternative splicing of pre-mRNA is an important mechanism for post-transcriptional regulation of gene expression and has increasingly been appreciated as a major mechanism to generate diversity of gene products in higher eukaryotes. 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 can lead to a number of cellular dysfunctions found in human and animal diseases (1,2). Using various model systems of regulated alternative splicing, exonic and intronic enhancers as well as silencers have been defined in pre-mRNAs. RNA-binding proteins which can be recruited to these RNA elements have also been identified (3). Moreover, a number of new technologies have been developed for genome-wide analysis of alternative splicing. Genome-wide splice array and computational analysis of whole genome sequences have defined a number of potential cis-elements for splicing regulation (4–6). Combinations of splice arrays, systemic identification of RNA targets for the RNA-binding splicing factors and high-throughput sequencing have been used to make a genome-wide splice map for Nova, Fox-2 and polypyrimidine tract binding protein (PTB), which relates the position of the target element of the RNA-binding protein to the splicing patterns (7–9). These studies have helped to predict splicing patterns of given genes. However our understanding of the molecular mechanism by which RNA-binding proteins regulate the splicing process is limited to SR proteins and some of the hnRNP proteins. Exonic enhancers and their binding proteins, SR proteins, have been well studied. For example, the interactions of the SR proteins with snRNP components and other basic pre-spliceosomal and spliceosomal proteins have been demonstrated during progression of the splicing steps (10,11). Studies on intronic and exonic silencers and their binding proteins such as PTB have provided several models for splicing repression (12,13). However, how RNA-binding proteins, which are recruited to intronic enhancers and often do not belong to SR proteins and hnRNP proteins, activate splicing of alternative exons is largely unknown. Only a few studies have
addressed the question of connecting the RNA enhancer binding proteins and splicing machinery (14,15).

One of the intronic enhancer elements which is involved in cell type or tissue-specific regulation of alternative splicing is the UGCAUG element. The importance of this element has been demonstrated in a number of cases of alternative splicing specific to neural cells, muscle cells and other cell types (16–19). This UGCAUG element does not necessarily function as an enhancer, but it enhances inclusion of alternative exons when it resides in an intron downstream of regulated exons (8). Jin et al. discovered that a zebrafish homolog of Caenorhabditis elegans Fox-1 could bind to this element in a highly sequence-specific manner (20). Subsequently a human homolog of Fox-1 has been shown to duplicate this property (21) and the solution structure of the RNA-binding domain of human Fox-1 in a complex with UGCAUGU has been determined (22). Identification of Fox-1 as an UGCAUG G-binding protein has had a major impact on the field of alternative splicing. Several laboratories have demonstrated that mammalian homologs of Fox-1 do indeed regulate alternative splicing via the UGCAUG element using model systems (20,21,23–27). Further, the unusually high sequence specificity of the Fox-1 target sequence has attracted investigators in bioinformatics to conduct genome-wide analysis for the location of the UGCAUG element and the splicing patterns of alternative exons (8,28).

In mammals, three genes are recognized as homologs of C. elegans Fox-1. Two of them were originally cloned in contexts other than splicing and the third gene was recognized after whole genome sequences had been determined (29–31). Different names were used for one of these genes in the literature. In this report, we use the nomenclature of Fox-1, Fox-2 and Fox-3. Fox-1 is also called A2BP1 and Hrnbp1 and Fox-2 is also called Rbm9, Fxh, Hrnbp2 and RTA, Fox-3 is called Hrnbp3, D11Bwg0517e and NeuN. All three Fox-1 family (Fox) proteins contain a single almost identical RNA recognition motif (RRM) in the central region of the molecule. Fox-1 is selectively expressed in brain and striated muscles, whereas Fox-2 is expressed in various tissues including brain and muscles (20,29,30). Both the Fox-1 and 2 genes generate tissue-dependent as well as tissue-independent isoforms by alternative splicing and alternative promoters. Different isoforms show different subcellular distributions and have quantitatively different effects on splicing (23,26,32). Fox-3 is expressed exclusively in neural tissues (31,33). Recently we have identified NeuN as the Fox-3 gene product (33). NeuN was originally defined by its immuno-reactivity with the monoclonal anti-NeuN antibody (34) and has been considered as a post-mitotic neuron-specific nuclear marker for over 15 years.

We have been studying the regulatory mechanisms of neural cell-specific alternative splicing using human nonmuscle myosin heavy chain II-B (NMHC II-B, named MYH10 in the human genome) as a model system. NMHC II-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 skipped in those from all other cells in mammals and birds (35,36). We have previously defined an intronic distal downstream enhancer (IDDE) which is located 1.5 kb downstream of N30. The IDDE confers neural cell-specificity on N30 inclusion and contains two copies of the UGCAUG element (17,37). We have shown that exogenously expressed brain isoforms of Fox-1 and 2 lead to N30 inclusion in the mRNAs derived from minigenes as well as from the endogenous NMHC II-B gene in cultured cells. The muscle-specific isoforms of Fox-1 and 2 demonstrate less activity than the brain isoforms on N30 splicing (23). Exogenously expressed Fox-3 isoforms also enhance N30 inclusion (33).

In this study, we analyze the expression pattern of the endogenous Fox proteins and the N30 splicing of endogenous NMHC II-B at the cellular level in mouse brain and spinal cord and we address a potential correlation of Fox-3 expression and N30 inclusion. Further, we identify Fox-3 interacting proteins as a step toward understanding the mechanism by which Fox-3 promotes inclusion of the alternative exon via binding to a downstream intron.

**MATERIALS AND METHODS**

**Generation of Fox-1, 2 and 3 antisera and other antibodies**

The N-terminal amino acids 1–116 and 1–112 of Fox-1 and Fox-2, respectively, fused to GST were expressed in BL21 bacteria and purified by a GSTrap FF column (GE Healthcare). GST-fused Fox-1 and Fox-2 proteins were used to generate polyclonal anti-Fox-1 and anti-Fox-2 in rabbits (Biosynthesis, Inc). The rabbit polyclonal anti-Fox-3 raised against the N-terminal amino acid 1–97 residues of Fox-3 was described previously (33). The other primary antibodies (Abs) used in this study were mouse monoclonal anti-NeuN (Millipore), mouse monoclonal anti-myc (Invitrogen), mouse monoclonal anti-GAPDH (Biosystems), mouse monoclonal anti-PB-associated splicing factor (PSF; Sigma), rabbit polyclonal anti-PSF (Santa Cruz Biotechnology), goat polyclonal anti-NonO (Santa Cruz Biotechnology), goat polyclonal anti-hnRNP-A3 (A-15, Santa Cruz Biotechnology) and rabbit polyclonal anti-NMHCII-B (38).

**Affinity chromatography and mass spectrometry**

For preparation of anti-Fox-3 immobilized resin, affinity purified polyclonal anti-Fox-3 was crosslinked to protein A/G agarose (Santa Cruz Biotechnology) with di-succinimidyl suberate (Sigma) according to an antibody crosslinking method (39). Nuclear extracts containing 5 mg of protein in 10 ml Co-IP buffer (Pierce) were incubated with the anti-Fox-3 crosslinked protein A/G agarose overnight at 4°C. The immuno-complexes were washed in Co-IP buffer. The complexes were solubilized in SDS sample buffer and subjected to SDS-PAGE. Following Coomassie blue staining, the protein bands were digested in the gels with trypsin. The recovered peptides were analyzed using an Applied Biosystems 4700
MALDI-TOF/TOF to acquire tandem MS/MS spectra. A compiled protein database was searched for the peptide sequences.

**FACS analysis**

Brain tissues were dissected and passed through a cell strainer (pore size, 40 μm) to prepare a single-cell suspension. The cells were washed in PBS containing bovine serum albumin, an RNase inhibitor (Roche) and a protease inhibitor cocktail (Sigma) and then permeabilized in a permeabilization buffer (eBioscience). Nonspecific binding sites were blocked with 5% goat serum for 30 min at 4°C, and then the cells were labeled with anti-NeuN at a final dilution of 1:100 for 45 min at 4°C. The cells were further incubated with Alexa-488 conjugated goat Abs against mouse IgG (Molecular Probes) at a 1:500 dilution for 15 min at 4°C. The specimens were examined using a Zeiss Axiophot microscope.

**In situ hybridization**

N30 included NMHC II-B mRNA was detected using a biotinylated oligonucleotide probe (Bioneer Inc.) complementary to the N30 region, 5’-CTGGGGTTTCACGGG CTTAGGGATTCCTG-3’. Hybridization and detection were carried out using an ISHyb In Situ Hybridization kit (Biochain Institute Inc.) and a DNA Detector System (KPL Inc.) according to the manufacturers’ protocols. The specimens were examined using a Zeiss Axiophot microscope.

**GST pull-down assay**

GST–Fox-3 fusion proteins were expressed in KRX bacteria cells and purified on glutathione sepharose beads (Amersham Pharmacia). The myc-tagged PSF and NonO were synthesized in vitro from the pCS3+MT constructs using the TNT Coupled Reticulocyte Lysate System (Promega). For GST pull-down assays, in vitro synthesized myc-tagged protein and GST-fusion protein bound to glutathione beads were incubated in 5–10 ml of PP-300 buffer (20 mM Tris–HCl pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 0.2% NP-40, 0.5 mM DTT, protease inhibitors) for 1 h at 4°C. The protein complexes were recovered in the SDS sample buffer. Following SDS-PAGE, the gels were stained with Coomassie blue or subjected to immunoblot analysis.

**RNA–protein UV crosslink**

The PCR products containing the T7 promoter upstream of the IDDEs were used as templates for RNA transcription (23). The RNA probes were synthesized by T7 RNA polymerase in the presence of [β-32P]UTP using a MAXiScript kit (Ambion). The myc-tagged Fox-3-L and PSF proteins were synthesized in vitro from pCS3+MT constructs using the TNT Coupled Reticulocyte Lysate System. Binding reactions were carried out in a 25 μl mixture that contains 10 mM HEPES (pH 7.9), 2 mM MgCl2, 1 mM ATP, 20 mM creatine phosphate, 50 ng yeast tRNA, 2 mM DTT, 2% polyethylene glycol (M.W. 3,550), 4 μl of the reticulocyte lysate reaction mixture and 1 x 104 cpm RNA probe for 20 min at 30°C. Reaction mixtures were irradiated with UV light (Stratalinker, Stratagene) for 20 min on ice, digested with 2 μl RNase-A/T1 (Ambion) and immunoprecipitated with 2 μg of anti-myc. Samples were subject to SDS-PAGE followed by autoradiography. The radioactive bands were quantified using a phosphorimagery STORM 860 (Molecular Dynamics).

**RESULTS**

**Distribution of Fox-1, 2 and 3 in the mouse central nervous system**

Fox-1, 2 and 3 are expressed in a tissue-dependent manner and neural tissues are the only tissues where all three Fox mRNAs are expressed (Supplementary Figure S1A). To analyze the distribution of the three Fox proteins at a cellular level in the central nervous system, the brain and spinal cord of postnatal day 10 mice were stained with rabbit polyclonal Abs. These Abs were newly produced in this laboratory against the N-terminal one-third of each Fox protein. The specificities of the Abs were verified by reactivity to recombinant Fox proteins expressed in cultured cells (Supplementary Figure S1C) and to mouse tissue extracts using immunoblots (Supplementary Figure S1B). In tissue extracts, 1–3 bands migrating at a molecular mass range of 30–55 kDa are detected using each Ab. Each Ab detects the proteins in only the tissues where the mRNAs are expressed, indicating the specificity. We have recently identified Fox-3 as NeuN, the antigen recognized by the widely used monoclonal Ab, anti-NeuN (33). Taking advantage of the fact that anti-NeuN is a mouse monoclonal Ab, tissue sections were stained with one of the polyclonal Fox Abs and anti-NeuN. Consistent with the fact that Fox-3 is NeuN, the anti-Fox-3 staining and the anti-NeuN staining overlap exactly in every single cell (Figure 1 E, J, O, and T). Co-staining with anti-Fox-1 and anti-NeuN and co-staining with anti-Fox-2 and anti-NeuN demonstrate overlapping staining as well as unique staining for each Ab (Figure 1).

In the hippocampal dentate gyrus, Fox-3 as well as NeuN expression is limited to the granular cell layer (GCL, Figure 1E), whereas Fox-2 expression is higher in the subgranular zone (SGZ) than the GCL (Figure 1C). Fox-1 is uniformly expressed in both the GCL and SGZ.
These differential expressions of Fox-1, 2 and 3 are further demonstrated by overlay images with NeuN staining (Figure 1B, D and E). In the cerebellum, Fox-3/NeuN staining is limited to the internal granular layer (IGL, Figure 1J). Anti-Fox-1 and anti-Fox-2 also weakly stain the IGL cells and both strongly stain Purkinje cell nuclei as well as the nuclei of interneurons in the molecular layer (ML) and Golgi cells (Figure 1F–I). In addition, Fox-2 is also detected in the inner layer of cells in the external germinal layer (EGL, Figure 1I, bracket) and the migrating granular cells (Figure 1H and I). This unique staining pattern of anti-Fox-2 suggests that Fox-2 is expressed in early post-mitotic neurons in addition to mature neurons. In the brain stem region, there is a mixture of Fox-3 only expressing cells, Fox-3 and Fox-1 or 2 expressing cells and Fox-1 or 2 expressing cells (Figure 1K–O). However, the expression pattern of Fox-1 is difficult to compare with that of Fox-2 in this region. In the spinal cord, motor neurons in the ventral horn express all three Fox proteins and other neurons in this area express them at a much lower level (Figure 1P–T). In summary, all three Fox proteins are expressed widely in the central nervous system, but there are differences in the relative levels of expression of each Fox protein. Fox-3 expression is absent in some specific neurons. All Fox proteins are localized predominantly to neuronal cell nuclei. In large neurons such as motor neurons in the spinal cord and brain stem, however, Fox-1 and Fox-3 can also be detected in soma.

Fox-3 expression and neural cell-specific alternative splicing of N30 in NMHC II-B

Next, we asked whether the differences in expression levels of Fox proteins among different neuronal cells have any relation to the splicing patterns of some pre-mRNAs. Because Fox-3, unlike Fox-1 and 2, is expressed only in neural tissues and Fox-3 expression seems to be restricted to certain types of neurons, brain cells
were dissociated and sorted into Fox-3 positive and negative cells by FACS using the monoclonal anti-Fox-3 (anti-NeuN). As shown in Figure 2A, 77.4% of dissociated cells from the cerebellum are Fox-3 positive. The majority of these cells shows low side scattering (SSC, granularity), consistent with being relatively small internal granular cells. Figure 2B shows that 42.5% of the cells dissociated from the brain stem and spinal cord are Fox-3 positive and the mean fluorescence intensity of these cells is 2- to 3-fold higher than those of cerebellar cells. Successful sorting by Fox-3 expression was verified by immunoblot analysis as shown in Figure 2C. This immunoblot confirms not just the presence or absence of Fox-3 but also shows that the average expression level of Fox-3 in the brain stem and spinal cord is higher than in the cerebellum. Of note is that the average expression levels of Fox-1 and 2 are similar between the Fox-3 positive and negative cell groups in the cerebellum, brain stem and spinal cord.

We have been studying neural cell-specific alternative splicing of pre-mRNA using NMHC II-B as a model. A cassette exon, N30, can be included only in the mRNA from some types of neural cells. We previously showed that the IDDE containing two UGCAUG elements is essential for N30 inclusion (17). Therefore, to determine whether N30 inclusion correlates with Fox-3 expression, the extent of N30 inclusion in the NMHC II-B mRNA was compared between Fox-3 positive and negative cell populations. Strikingly, Fox-3 positive cells from the brain stem and spinal cord include N30 to a much greater extent than Fox-3 negative cells (Figure 2D, lanes 3 and 4). Fox-3 positive cells from the cerebellum, which express Fox-3 at a lower level than those from the brain stem and spinal cord, also include N30 to a small extent (Figure 2D, lane 2). In contrast, Fox-3 negative cells almost completely skip N30 (Figure 2D, lanes 1 and 3), despite the fact that Fox-1 and 2 in Fox-3 negative cells are expressed at similar levels to Fox-3 positive cells (Figure 2C).

We also analyzed the relation between Fox-3 expression and N30 inclusion using tissue sections. NMHC II-B is expressed ubiquitously except in a few cell types such as hematopoietic cells. In neural tissues, Purkinje cells in the cerebellum and motor neurons in the spinal cord express especially high levels of NMHC II-B, as shown in Figure 3A (red, arrowheads in a and arrows in b). However Fox-3 is expressed in motor neurons (Figure 3A-b, green, arrows) but not in Purkinje cells (Figure 3A-a, arrowheads). Instead, Purkinje cell nuclei contain Fox-1 and 2 (Figure 1F–I). In situ hybridization analysis using an antisense oligonucleotide probe complementary to the N30 sequence demonstrates that N30 expression is robust in motor neurons (Figure 3B-b, arrows) but is not detected in Purkinje cells. Sense oligonucleotide probe does not give any signal (data not shown). These biochemical and histological analyses

![Figure 2](https://example.com/f2.png)

**Figure 2.** Inclusion of N30 in NMHC II-B mRNAs preferentially occurs in Fox-3 expressing cells. (A, B) FACS analysis of the cells dissociated from mouse cerebellum (A) and brain stem and spinal cord (B) using the monoclonal anti-Fox-3 (anti-NeuN). The numbers inside each panel represent percentage of cells in each pool. The dotted line represents the mean fluorescence intensity. SSC, side scattering. (C) Immunoblot analysis of Fox-3 positive (+) and negative (−) cells sorted by FACS. Abs used in each blot are indicated on the right. GAPDH serves as a loading control. (D) N30 splicing patterns in Fox-3 positive (+) and negative (−) cells sorted by FACS. Ethidium bromide stained agarose gels of the RT-PCR products are shown. The upper and lower bands include and exclude N30, respectively. Numbers shown between the agarose gels indicate percentage of the N30 inclusion.
support a positive correlation between N30 splicing and Fox-3 expression.

We have previously shown that all three Fox proteins are capable of activating N30 inclusion when they are over-expressed in cultured cells (23,33). There are always some concerns about the relevance of over-expression experiments. Therefore, we asked whether the endogenous level of Fox-3 expression is sufficient for N30 splicing. Embryonic carcinoma-derived P19 cells express Fox-3 during neural differentiation triggered by retinoic acid treatment, whereas undifferentiated P19 cells do not express Fox-3 (33). Immuno blot analysis confirms that Fox-3 expression is induced and also shows that the Fox-2 expression level is unchanged before and after differentiation (Figure 4A). Fox-1 is barely detected under these culture conditions. These cells were transfected with the minigene reporter construct which contains exon N30 and the flanking exons E5 and E6, and introns of the human NMHC II-B gene (Figure 8A). N30 splicing patterns were then analyzed. Upon retinoic acid treatment, induction of Fox-3 is accompanied by an increase in N30 inclusion from 12 to 56% (Figure 4C, lanes 1 and 2). Next T-2 and T-3 shRNAs, which target two different regions of Fox-3 mRNAs, were used to inhibit Fox-3 expression (Figure 4B, lanes 2–4). GFP shRNA served as a negative control. The shRNA (T-2 and T-3)-mediated reduction of Fox-3 results in a decrease in N30 inclusion from 54 to 24% (Figure 4C, lanes 3–5). Rescue experiments shown in Supplementary Figure S2B rule out off-target effects of the T-2 shRNA. Since differentiated P19 cells express Fox-2 in addition to Fox-3 (Figure 4A), whether Fox-2 is required for N30 inclusion was tested.

Knock-down of Fox-2 expression by siRNA does not affect N30 inclusion (Figure 4D). These results indicate that the endogenous level of Fox-3 causes a significant enhancement of N30 splicing, whereas the endogenous level of Fox-2 is not sufficient and even not required for N30 inclusion in neurally differentiated P19 cells.

Identification of PSF as an interacting protein with Fox-3

To understand the mechanism responsible for Fox-3-mediated regulation of alternative splicing, we searched for nuclear factor(s) which interact with Fox-3. We isolated protein complexes containing Fox-3 from nuclear extracts of mouse brains by affinity chromatography using affinity purified anti-Fox-3 covalently coupled to a resin. Following SDS-PAGE, mass spectrometry analysis of the protein bands revealed that the Fox-3 complexes contain several proteins. We focus on PTB-associated splicing factor (PSF) and non-POU domain-containing octamer-binding protein (NonO) in this study. Mass spectrometry data are shown in Supplementary Figure S3. We next analyzed the Fox-3-containing complexes by co-immunoprecipitation using nuclear extracts from differentiated P19 cells. Consistent with the results from affinity chromatography, co-immunoprecipitation with anti-Fox-3 followed by immunoblots shows that Fox-3 can associate with PSF and NonO (Figure 5A). Although PSF and NonO contain RRM s (40), the interaction of these proteins with Fox-3 is not mediated by RNA as demonstrated in Figure 5A (lanes 3 and 4). RNase treatment does not disrupt the interaction of Fox-3 with PSF and NonO. On the other hand, the association of hnRNP-A3 with Fox-3 is disrupted by RNase treatment, indicating that the Fox-3 and hnRNP-A3 complex is mediated by RNA. Reciprocal co-immunoprecipitation using anti-PSF or anti-NonO followed by immunoblotting further establishes that Fox-3, PSF and NonO form a complex (Figure 5B and C).

To determine whether the interaction of Fox-3 with PSF and NonO is direct or indirect and to determine regions of the proteins responsible for the interaction, in vitro binding assays using recombinant proteins were carried out. It is known that the Fox-3 gene generates multiple isoforms by alternative splicing (33,41). Two isoforms Fox-3-L and Fox-3-S, which include and exclude 47 amino acids in the C-terminal region, respectively, were used for these experiments. The bacterially expressed and purified GST-fusion proteins containing full-length and various domains of Fox-3 (Figure 5D) were incubated with myc-tagged PSF or NonO, which were generated by in vitro transcription–translation in reticulocyte lysates. As shown in the upper panel of Figure 5E, full-length Fox-3-L and S and the C-terminal C-L and S regions interact directly with PSF, but the N-terminal region and the RRM of Fox-3 do not. We also generated various domains of PSF (Figure 5F). As seen in Figure 5G, the N-terminal region including the P/Q rich domain interacts with the Fox-3 C-terminal region. Notably, NonO does not interact directly with any region or with full-length Fox-3 (Figure 5E, middle panel). Since
a number of studies have previously reported the direct interaction of PSF and NonO (42,43), it is likely that NonO is recruited to the Fox-3 complex via PSF in native tissue and cell extracts.

Binding activities of PSF to Fox proteins and the effects of PSF on the Fox-3 and target RNA interaction

We next tested the binding activity of PSF to each Fox member. Fox-1 and 2 have a number of tissue-dependent and tissue-independent isoforms generated by alternative splicing. We chose the isoforms which are expressed in brain and contain similar C-terminal sequences to that of Fox-3. In vitro translated PSF and each of the Fox proteins, all of which were tagged with the myc epitope, were incubated and immunoprecipitated with anti-PSF. Following SDS-PAGE, the blots were immuno-stained using anti-myc. Quantification of myc signals provides the ratio of myc-Fox, which was co-immunoprecipitated with PSF, to myc-PSF, which was immunoprecipitated by anti-PSF regardless of its association with Fox proteins. Under given conditions, PSF pulls down Fox-3-L and Fox-3-S 2.7 fold more efficiently than Fox-2 and 7 fold more efficiently than Fox-1 (Figure 6A). These results suggest that PSF might have the highest affinity for Fox-3 among the Fox proteins.

We also examined whether interaction of Fox-3 with PSF has any effect on the binding activity of Fox-3 to its target RNA sequence. Radiolabeled IDDE of the NMHC II-B gene which contains two copies of the UG CAUG element (wt-IDDE) or a mutant form of this element (mc-IDDE, see Figure 8A) was mixed with myc-tagged Fox-3 or myc-tagged PSF or with both myc-tagged proteins, crosslinked by UV and subjected to immunoprecipitation with anti-myc. Both Fox-3 and PSF could be immunoprecipitated regardless of their interaction and the radiolabeled RNA crosslinked to the proteins was visualized (Figure 6B). As expected, Fox-3 binds to wild-type IDDE but not to mutant mc-IDDE. No regions of the IDDE are crosslinked with PSF, indicating that PSF does not bind directly to the IDDE. Of note, however, the quantification of radioactivity by a phosphorimager shows that Fox-3 binds to the UGCAUG element in the IDDE more efficiently (2- to 3-fold) in the presence of PSF, suggesting that the Fox-3-PSF complex has a higher affinity for the UG CAUG element compared to Fox-3 alone. Since our crosslinking conditions do not crosslink Fox-3 and PSF, a protein complex with the expected larger molecular mass with radiolabeled RNA is not detected. Similar experiments were carried out using pre-mRNA which was composed of exons E5, N30 and E6 and the truncated introns including the wild-type or mutant IDDE. Essentially the same results were obtained (data not shown).
PSF is an essential coactivator of Fox-3 for N30 splicing

To understand the functional consequences of the interaction of Fox-3 with PSF and NonO, we studied the effects of exogenous expression of these proteins on N30 splicing of the minigene in SK-N-SH cells. As shown in Figure 7A, exogenous expression of Fox-3-L or S enhances N30 inclusion (lanes 2 and 3). Interestingly, exogenous expression of PSF alone also enhances N30 inclusion whereas NonO does not (Figure 7A, lanes 4 and 5). Expression of both Fox-3 and PSF shows an apparently additive effect on N30 splicing (Figure 7A, lanes 6 and 8, see ‘Discussion’ section). Fox-3 together with
NonO or PSF together with NonO shows essentially the same effect on N30 inclusion as that of Fox-3 alone or PSF alone (Figure 7A, compare lanes 7, 9 and 10 to lanes 2 and 4). When all three proteins, Fox-3, PSF and NonO, are co-expressed, the increase in N30 inclusion is similar to that found in the absence of NonO (Figure 7A, compare lanes 11 and 12 to lanes 6 and 8). These results suggest that the interaction of Fox-3 with PSF (and NonO) results in the cooperative enhancement of N30 inclusion. This notion is further supported by experiments shown in Figure 7B and C. The cooperative enhancement of N30 splicing by PSF (and NonO) with Fox-3 can be observed when the C-terminal region of Fox-3 is intact. The N-terminal-RRM of Fox-3 does not show the enhancement effect. These observations are consistent with the fact that PSF interacts with the C-terminal region of Fox-3.

As shown in Figure 7A, lane 4, exogenous expression of PSF can activate N30 splicing in the absence of Fox-3. We asked whether PSF-dependent N30 inclusion depends on the downstream UGCAUG element. Mutation of either one or both UGCAUG elements results in a significant decrease in PSF-dependent N30 inclusion (Figure 8B, lanes 2–4). The host SK-N-SH cells do not express endogenous Fox-3 or Fox-1 at a detectable level, but they endogenously express Fox-2. As already shown in Figure 6A, PSF can interact with Fox-2 though less efficiently than with Fox-3. Therefore we investigated whether endogenous Fox-2 participates in PSF-induced N30 inclusion. Knock-down of Fox-2 expression by siRNA abolishes PSF-induced activation of N30 splicing as well as basal splicing (Figure 8C). Conversely, we also asked whether endogenous PSF participates in Fox-3-induced activation of N30 splicing. Knock-down of the endogenous PSF by siRNA also abolishes Fox-3-induced N30 splicing as well as basal splicing almost completely (Figure 8D). We also tested whether or not PSF is required for N30 splicing which is induced by endogenous Fox-3 expression in P19 cells during neural differentiation. Knock-down of PSF expression by siRNA abolishes N30 inclusion in differentiated P19 cells (Figure 8E). Therefore, both endogenous Fox-3 and PSF are required for N30 splicing in these cells. Furthermore immunostaining of the mouse spinal cord shows co-localization of Fox-3 and PSF, especially in motor neurons in the ventral horn where N30 is robustly expressed (Supplementary Figure S4). This is consistent with the notion that N30 splicing requires both Fox-3 and PSF. Together all these results indicate that PSF is an essential coactivator of Fox-3 for N30 splicing. PSF and Fox-3/Fox-2 directly interact and cooperatively activate N30 splicing. Activation of N30 splicing by one of the two factors (PSF and Fox-3/Fox-2) is dependent on the presence of the other factor.

Human NMHC II-B pre-mRNA is ~156 kb in length and contains 41 constitutive and 3 alternative exon sequences. The IDDE region is located 1.5 kb downstream of N30 in the native pre-mRNA. The endogenous pre-mRNA is much more complex than pre-mRNAs from the minigenes. Therefore we examined whether the above observation obtained using minigenes is comparable with N30 splicing of the endogenous NMHC II-B pre-mRNA in human cells. SK-N-SH cells were transiently transfected with different combinations of expression constructs for Fox-3, PSF and NonO and the splicing patterns of N30 were analyzed. As shown in Figure 9A...
(lane 4), inclusion of N30 in the endogenous mRNA is markedly increased by simultaneous exogenous expression of Fox-3, PSF and NonO. We also analyzed the effects of PSF on the recruitment of Fox-3 to the IDDE region in the endogenous pre-mRNA. Since SK-N-SH cells express endogenous PSF, the cellular level of PSF was either decreased by siRNA or increased by exogenous expression of PSF. RNA–protein co-immunoprecipitation using the monoclonal anti-Fox-3 (anti-NeuN) and cellular extracts followed by RT-PCR for the IDDE region demonstrates that the recruitment of exogenously expressed Fox-3 to the IDDE depends on the cellular concentration of PSF (Figure 9B). The lowest panel of Figure 9B shows quantification of RT-PCR by real-time PCR. siRNA-mediated reduction of endogenous PSF reduces the amounts of the IDDE associated with Fox-3 about 4-fold. Exogenous expression of PSF leads to a 30-fold increase in Fox-3 binding to the IDDE compared to the PSF-knockdown. Levels of Fox-3 expression and changes in the levels of PSF among a set of transfections were

Figure 7. Cooperative activation of N30 splicing by Fox-3 and its interacting proteins PSF and NonO. (A) Effects of Fox-3, PSF and NonO on N30 splicing. The NMHC II-B minigene was co-transfected into SK-N-SH cells with the expression constructs using the different combinations indicated. The N30 splicing patterns of the minigene mRNAs were analyzed by RT-PCR (upper panel). The exogenously expressed proteins were verified by immunoblots using anti-myc (lower panel). (B) Schematic representation of full-length and deletion constructs of Fox-3 tagged with the myc epitope. (C) The C-terminal region of Fox-3 confers cooperativity with PSF and NonO on activation of N30 splicing. The experiment was performed similar to A.
verified by immunoblots shown in Figure 9C. Taken together, the increased Fox-3 binding to the IDDE by PSF is accompanied by increased N30 splicing of the endogenous pre-mRNA. Interaction of Fox-3 and PSF is an integral part of the mechanism responsible for the Fox-3-dependent activation of N30 splicing.

DISCUSSION

In this study, we identified PSF as a Fox-3 interacting protein and demonstrated that this interaction is essential for Fox-3 to activate neural cell-specific alternative splicing of the N30 exon of NMHC II-B. We also compared the expression pattern of Fox-3 to those of Fox-1 and 2 at the cellular level in the mouse central nervous system and found a correlation between Fox-3 expression and N30 splicing.

Two laboratories have reported the tissue distribution and subcellular localization of Fox-1 in the mammalian nervous system (24,29). Although they agreed that Fox-1 is expressed in neuronal cells, there is a discrepancy as to whether it localizes to the nucleus or cytoplasm. Our observations largely agree with that of Black and colleagues that Fox-1 localizes predominantly to nuclei. However, we also detected Fox-1 in the somas and nuclei of motor neurons in the spinal cord. Discrepancies in histological observations might be due in part to differences in the age
Figure 9. Fox-3 and PSF cooperatively enhance N30 inclusion in endogenous NMHC II-B mRNAs by recruitment of Fox-3 to the IDDE. (A) Enhancement of N30 inclusion in endogenous NMHC II-B mRNA by Fox-3 and its interacting proteins PSF and NonO. SK-N-SH cells were transiently transfected with the expression constructs for myc-tagged Fox-3-L, PSF and NonO with the different combinations indicated. The N30 splicing patterns of the endogenous NMHC II-B mRNAs were analyzed by RT-PCR. The exogenously expressed proteins were verified by immunoblot using anti-myc. (B, C) PSF-dependent recruitment of Fox-3 to the IDDE of the endogenous NMHC II-B transcript. SK-N-SH cells were transfected with the expression constructs and siRNA in different combinations as indicated. An empty vector and a non-targeting siRNA were used as negative controls (−). The cell extracts were subject to RNA-protein co-immunoprecipitation using the monoclonal anti-Fox-3 (anti-NeuN). The IDDE region of the NMHC II-B transcripts associated with Fox-3 was analyzed by RT-PCR. The end products of RT-PCR are shown following agarose gel electrophoresis (B, middle panel, lanes 6–9). Quantification by real-time PCR is shown in the lowest panel. The presence of equal amounts of the IDDE transcript among a set of the transfected cells was verified using cell extracts without immunoprecipitation (B, lanes 1–4). Immunoblot analysis of the input samples verifies changes in amounts of the expressed proteins (C). ND, not detectable; Endo-PSF, endogenous PSF.

and species of the animals, differences in methods for tissue fixation and antigen retrieval and differences in the epitopes recognized by the Abs. Of interest, it has been reported that the depolarization-induced change in the Fox-1 isoform results in a change in the nucleo-cytoplasmic ratio of Fox-1 (32). We have also previously reported that different isoforms of Fox-1 show different subcellular localizations when they are exogenously expressed in cultured cells (23). Therefore, it may be necessary to determine which isoform of Fox-1 is dominant in each particular system.

Our co-immunostaining analysis and FACS followed by immunoblotting demonstrated that some neuronal cells expressing Fox-1 or 2 do not express Fox-3. Of note is that the expression levels of Fox-3, but not Fox-1 or 2, correlate with the N30 splicing patterns in the cerebellum, brain stem and spinal cord. Most of the previous studies on brain specific alternative splicing have used whole brain or anatomically dissectible parts of the brain. We made use of FACS to separate cells according to the expression levels of Fox-3. A combination of cell sorting and biochemical analysis led us to find different splicing patterns between the Fox-3 positive and negative cell populations. In spite of our previous observation that exogenous expression of Fox-1 or 2 enhances N30 inclusion in cultured cells (23), brain cells which express Fox-1 and/or Fox-2 but not Fox-3 do not activate N30 splicing. This could be explained in part by the following. Since the average expression level of Fox-1 or 2 is similar between Fox-3 positive and negative cells in the cerebellum, brain stem and spinal cord, additional expression of Fox-3 simply increases the total amounts of all three Fox proteins. Another reason might be related to the isoforms of Fox-1 and 2. Fox-1 and 2 genes generate multiple alternatively spliced isoforms. Differences in amino acid sequences at the C-terminal region result in significant differences in the splicing activity toward N30 (23). Currently our study does not distinguish differences in the C-terminal amino acids, since anti-Fox-1 was generated against the N-terminal region common to Fox-1 isoforms and the same is true for anti-Fox-2. Therefore, cells negative for Fox-3 might express Fox-1 and/or Fox-2 isoforms with lower splicing activities. A third reason might be a difference in affinity for interacting protein(s). A number of proteins including ataxin-1 and 2, atrophin-1, quaking, Fyn tyrosine kinase and estrogen receptor-α have been reported to interact with Fox-1 or Fox-2 in mammals (29,44–46). However, most of these proteins have not been functionally characterized in the context of pre-mRNA splicing. Of particular interest is a report showing the interaction of an U1 snRNP component, U1C protein, with Fox-1 and 2 in yeast two-hybrid screening (47), although the functional outcome of this interaction has not been studied. In this study, we identified PSF as an essential interacting protein of Fox-3 for activation of N30 splicing. Even though we compared the isoforms of Fox-1 and 2 which show the highest sequence similarity to Fox-3 and the highest activity for N30 splicing among the known isoforms, PSF binds to Fox-3 more efficiently compared to Fox-1 and 2. Therefore this could explain why Fox-3 is more active in N30 inclusion. This affinity difference also suggests that Fox-3 might play a role in the determination of neural specificity of alternative splicing. However differences in affinity of PSF for Fox proteins still need to be studied in greater detail biochemically and evaluated more precisely in the cellular context.
Although we found a good correlation between the level of Fox-3 expression and the extent of N30 splicing, this study does not exclude a possible contribution of Fox-1 and 2 to N30 splicing. Fox-1 and 2 have been reported to interact with each other using a yeast two-hybrid system (46). Our lab also detected the interaction of Fox-2 with Fox-3 using the yeast two-hybrid system as well as the interaction of Fox-3 with Fox-1 and with Fox-2 by co-immunoprecipitation (unpublished observations). In fact, we rarely observed neuronal cells which express only Fox-3. It was unexpected to find that Fox-1 localized predominantly to nuclei in intact brain, since all Fox-1 isoforms which we have analyzed were diffusely distributed in both the nuclei and cytoplasm or predominantly localized to the cytoplasm when they were exogenously expressed in cultured cells (23). In contrast, exogenously expressed Fox-3 isoforms localize almost exclusively to nuclei in cultured cells (Supplementary Figure SID). The Fox-2 isoforms which we have analyzed localized predominantly to nuclei (23). These observations raise the possibility that Fox-1 might dimerize with Fox-3 or Fox-2 and localize to nuclei in vivo. The Fox proteins might function as heterodimers or homomultimers, especially when the pre-mRNA contains multiple UGCAUG elements. Whether there is a difference in splicing activity among heterodimers and homodimers still needs to be determined.

Fox proteins can function as activators or repressors depending on their binding location on pre-mRNAs relative to the regulated exons. Recent genome-wide studies together with earlier studies using model systems have proposed a general rule for Fox proteins to influence the choice of exons (8,20). When Fox proteins bind to the intron downstream of the alternative exon, exon inclusion occurs. On the other hand, when Fox proteins bind to the intron upstream of the alternative exon, exon skipping occurs. Recently a few reports have begun to address the mechanism by which Fox proteins repress or activate the usage of alternative exons. Using F1α pre-mRNA, Fox-1 which is recruited to the upstream intron has been shown to block formation of the early pre-spliceosome complex on the intron downstream of the regulated exon (48). In the case of calcitonin/CGRP pre-mRNA, Fox-2 which is bound to the upstream intron inhibits the recruitment of SF1 at the branch site, and further, Fox-2 which is bound to the alternative exon inhibits the recruitment of U2AF at the 3’ splice site (27). The interaction of Fox proteins (FOX-1 and ASD-1) with another sequence-specific RNA-binding protein SUP-12, which is expressed specifically in muscle, has been reported in C. elegans. The FOX-1 (or ASD-1) and SUP-12 interaction enhances their binding to their adjacent target RNA elements on the egl-15 pre-mRNA and leads to inclusion of a muscle specific mutually exclusive exon. This report provides a mechanism for the strict tissue specificity of Fox-regulated alternative splicing (49).

In this study we identified PSF and the PSF–NonO complex as proteins interacting with Fox-3. The human ortholog of NonO is often called p54nrb. PSF and NonO are structurally related proteins that contain two RRM's and can bind to RNA as well as DNA (40). PSF and NonO interact with each other to form a heterodimer and the participation of PSF or the PSF–NonO complex has been reported in many aspects of nuclear function including transcription, transcriptional termination, pre-mRNA splicing, 3’ end processing of mRNA and RNA retention (40,50,51). PSF and NonO are expressed in various tissues and cell types. We demonstrated that the C-terminal region of Fox-3 binds directly to the N-terminal region of PSF, and Fox-3 and NonO interact indirectly via PSF. PSF enhances the binding of Fox-3 to the target UGCAUG element in an in vitro crosslinking assay. Moreover the presence of PSF enhances the recruitment of Fox-3 to the IDDE of the NMHC II-B transcript, which contains the UGCAUG elements, in intact cells. The effect of PSF on Fox-3 binding to the target RNA element in intact cells shows an apparently greater degree of enhancement (>-30-fold) than in vitro (2- to 3-fold). This difference could be due to the different efficiencies of the Fox-3 and PSF complex formation between in vitro and intact cells. Another possibility is that PSF and Fox-3 might be co-transcriptionally recruited to the Fox-3 target sites of pre-mRNAs. PSF and NonO have been reported to be associated with phosphorylated RNA polymerase II in a large complex containing transcriptional elongation factors (52,53). If Fox-3 was included in that complex via PSF, Fox-3 could be more efficiently recruited to target sites of pre-mRNAs during transcriptional elongation, compared to simple diffusion in the nucleus.

Although we showed that Fox-3 binding to the target RNA element was enhanced by PSF, the role of PSF in Fox-3-dependent activation of alternative splicing may not be limited to this effect. PSF was originally found as a spliceosome associated protein (54). A number of studies have demonstrated the presence of PSF in the pre-spliceosome and in the spliceosome at different stages and in complexes containing snRNPs (55). PSF has also been reported to interact directly with U5 snRNA and with the 5’ splice site under splicing conditions (42,53). Therefore PSF may function as a mediator between Fox-3-bound pre-mRNA and the splicing machinery. This notion is supported by our observation that PSF does not bind directly to the IDDE or to the pre-mRNA from the NMHC II-B minigene in the absence of other nuclear factors. Additionally, both Fox-3 (or other Fox proteins) and PSF are essential for the UGCAUG-dependent activation of N30 splicing. In the presence of endogenous PSF and Fox-2, exogenous Fox-3 activates N30 splicing to some extent as does exogenous PSF. These effects of exogenous Fox-3 and PSF are apparently additive. When endogenous Fox-2 or PSF is eliminated, however, neither exogenous Fox-3 nor PSF can activate N30 splicing. The enhancing effect of PSF on N30 splicing is dependent on the UGCAUG element, although it does not bind to this element, and is absolutely dependent on the presence of Fox-3 (or other Fox proteins). The enhancing effect of Fox-3 on N30 splicing is also absolutely dependent on the presence of PSF. Thus, the effects of the two proteins are actually not additive, but rather cooperative. PSF seems to function as a coactivator or mediator of Fox-3 during the splicing process. The simplest model to
accommodate all the data that is PSF or the PSF-containing complex bridges Fox-3 and the splicing machinery. Although the detailed molecular mechanism of N30 exon recognition following the Fox-3 and PSF interaction remains to be elucidated, this interaction has now been shown to be an integral part of the mechanism responsible for Fox protein regulated activation of alternative exon inclusion via a downstream intronic enhancer.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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