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A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing via the pentanucleotide GCAUG

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Alternative splicing is one of the central mechanisms that regulate eukaryotic gene expression. Here we report a tissue-specific RNA-binding protein, Fox-1, which regulates alternative splicing in vertebrates. Fox-1 bound specifically to a pentanucleotide GCAUG *in vitro*. In zebrafish and mouse, *fox-1* is expressed in heart and skeletal muscles. As candidates for muscle-specific targets of Fox-1, we considered two genes, the human mitochondrial ATP synthase γ-subunit gene (F1γ) and the rat α-actinin gene, because their primary transcripts contain several copies of GCAUG. In transfection experiments, Fox-1 induced muscle-specific exon skipping of the F1γ gene via binding to GCAUG sequences upstream of the regulated exon. Fox-1 also regulated mutually exclusive splicing of the α-actinin gene, antagonizing the repressive effect of polypyrimidine tract-binding protein (PTB). It has been reported that GCAUG is essential for the alternative splicing regulation of several genes including fibronectin. We found that Fox-1 promoted inclusion of the fibronectin EIIIB exon. Thus, we conclude that Fox-1 plays key roles in both positive and negative regulation of tissue-specific splicing via GCAUG.
Keywords: alternative splicing/Fox-1/GCAUG/RNA binding

Introduction

Alternative splicing leads to the generation of functionally distinct proteins from a single gene and often controls how a gene acts during development and differentiation (reviewed in Lopez, 1998; Smith and Valcarcel, 2000; Grabowski and Black, 2001; Graveley, 2001; Maniatis and Tasic, 2002). The *Drosophila* sex determination pathway is the most striking example of regulation involving a cascade of regulated alternative splicing events (for reviews, see Inoue *et al*., 1995; Lopez, 1998; Smith and Valcarcel, 2000). For example, Sex-lethal (Sxl) protein induces female-specific splicing of *transformer* (*tra*) pre-mRNA, blocking the use of the non-sex-specific acceptor site, resulting in generation of functional Tra protein only in females. Tra and Tra-2 proteins bind to the exonic regulatory sequences and promote female-specific splicing of *doublesex* (*dsx*) pre-mRNA. In vertebrates, many genes are regulated by alternative splicing. It has been estimated that 40–60% of the human gene transcripts are alternatively spliced (for reviews, see Black, 2000; Graveley, 2001; Lander *et al*., 2001). Despite the importance of alternative splicing, many questions about how it is regulated remain unanswered.

Recently, a few tissue-specific regulators of alternative splicing have been reported in vertebrates. A neuron-specific RNA-binding protein, Nova-1, regulates alternative splicing in neurons: *nova-1*-null mice have a specific splicing defect in the inhibitory glycine receptor α2 (GlyRα2) exon 3A (Jensen *et al*., 2000). QKI-5, encoded by the mouse *quaking* gene, regulates alternative splicing of myelin-associated glycoprotein (MAG) (Wu *et al*., 2002). CELF/Bruno-like proteins play important roles in nervous-specific splicing and muscle-specific splicing (Ladd *et al*., 2001; Charlet *et al*., 2002; Suzuki *et al*., 2002; Zhang *et al*., 2002).

It has been shown that ubiquitously expressed factors, such as the SR proteins and the heterogeneous nuclear ribonucleoproteins (hnRNPs), play important roles in alternative splicing (reviewed in Smith and Valcarcel, 2000). Polypyrimidine tract-binding protein (PTB), or hnRNP I, regulates the alternative splicing of many genes such as *c-src*, *FGF-R2*, *calcitonin/CGRP*, *GABA receptor γ2*, *α-tropomyosin* and *α-actinin* (for reviews, see Smith and Valcarcel, 2000; Wagner and Garcia-Blanco, 2001). PTB functions as a negative regulator of alternative splicing, binding to intronic splicing silencer elements. It is likely that negative regulation of alternative splicing by PTB is derepressed by tissue- and stage-specific factors.

Other clues to the mechanisms of alternative splicing have emerged from studies of a splicing enhancer element, (U)GCAUG. The hexanucleotide UGCAUG has been found to be required for EIIIB exon inclusion of fibronectin (Huh and Hynes, 1994; Lim and Sharp, 1998). Moreover, UGCAUG and/or GCAUG are essential for exon inclusion in the splicing regulation of some genes such as *c-src* (Modafferi and Black, 1997), *calcitonin/CGRP* (Hedjran *et al*., 1997), *non-muscle myosin heavy chain* (*NMHC*)*-B* (Kawamoto, 1996) and *4.1R* (Deguillien *et al*., 2001). A recent computational study on the human genome showed that UGCAUG often lies in flanking introns of brain-specific and muscle-specific exons (Brudno *et al*., 2001). Thus, it is highly likely that the GCAUG sequence plays key roles in tissue-specific

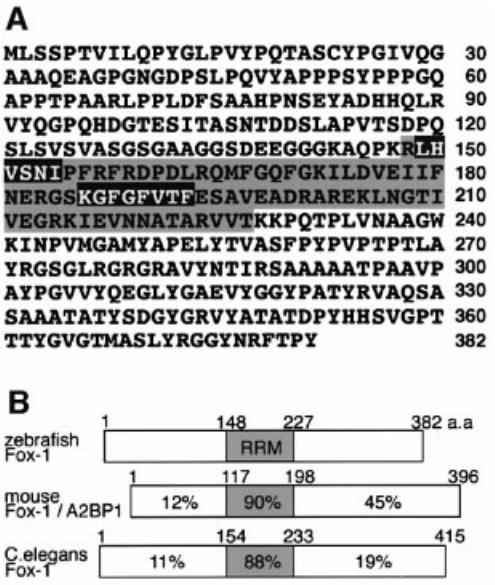

Fig. 1. Identification of zebrafish and mouse Fox-1 proteins. (**A**) Amino acid sequence of zebrafish Fox-1. The RNA recognition motif (RRM) is shaded (Burd and Dreyfuss, 1994). The RNP2 (hexamer) and RNP1 (octamer) motifs are boxed in black. (**B**) Schematic representation of zebrafish, mouse and nematode Fox-1 proteins. The percentage identity of amino acid sequences is shown.

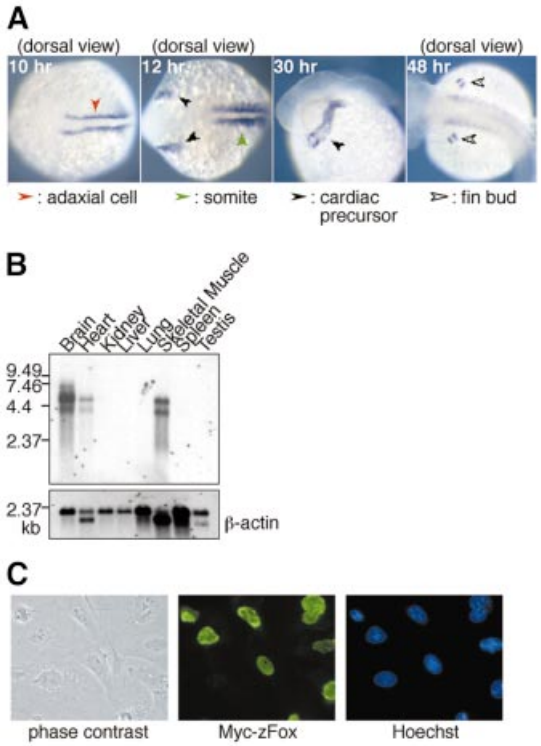

Fig. 2. Expression of zebrafish and mouse *fox-1* genes. (**A**) Whole-mount *in situ* hybridization of zebrafish embryos using a *fox-1* probe. A dorsal view of a 10 h embryo shows *fox-1* expression in adaxial cells, precursor cells of slow muscle. A dorsal view of a 12 h embryo shows *fox-1* expression in somites and bilateral presumptive heart cells. Expression in the developing heart as well as in somites is observed at 30 h. A dorsal view of a 48 h embryo shows expression in the finbud cells. (**B**) Northern blot analysis of mouse Fox-1 (upper) and β-actin (lower) using MessageMap Blot (Stratagene). (**C**) Nuclear localization of Fox-1 protein fused with a Myc tag was detected in CV-1 cells. Hoechst staining of the cells is shown in the right panel.

splicing. However, nothing is known about possible regulatory protein(s) that bind to GCAUG.

In the present study, we report a tissue-specific RNA-binding protein, Fox-1, which binds specifically to the GCAUG sequence. In zebrafish, *fox-1* is expressed during muscle development, whereas a mouse homologous gene is expressed in brain as well as heart and skeletal muscle. Here we show that Fox-1 promotes muscle-specific splicing via binding to GCAUG in transfection experiments. Our data suggest that Fox-1 plays key roles in both positive and negative regulation of tissue-specific splicing.

Results

fox-1 encodes a tissue-specific nuclear RNA-binding protein

In *Caenorhabditis elegans*, *fox-1* is involved in sex determination and dosage compensation, acting as a post-transcriptional repressor of the sex determination gene *xol-1* (Meyer, 2000). It has been suggested that nematode *fox-1* may regulate sex-specific splicing of *xol-1* (Skipper *et al*., 1999). We therefore expected that a Fox-1-related protein might function as a splicing regulator in vertebrates. Here we identified an RNA-binding protein, zebrafish Fox-1 (zFox-1), that contains an RNA recognition motif (RRM) (Burd and Dreyfuss, 1994) and has homology to nematode Fox-1 (Hodgkin *et al*., 1994) and human ataxin2-binding protein A2BP1 (Shibata *et al*., 2000) (Figure 1). Whole-mount *in situ* hybridization analysis showed that zebrafish *fox-1* was expressed specifically during muscle development: its mRNA was observed in adaxial cells, somites, cardiac precursors, fin buds and jaw muscle cells (Figure 2A; data not shown). Northern blotting analysis showed that mouse Fox-1/A2BP1 is expressed in heart, skeletal muscle and brain (Figure 2B), like human A2BP1 (Shibata *et al*., 2000). In addition, we found that both zebrafish and mouse Fox-1 proteins fused with a Myc tag were localized in the nucleus (Figure 2C; data not shown). These results suggest that Fox-1 is involved in nuclear RNA processing events in a tissue-specific manner.

Fox-1 binds to GCAUG in vitro

To identify target RNA molecules for Fox-1 protein, we performed an *in vitro* selection experiment using a zFox-1 protein fused with GST. After five rounds of selection and amplification, 18 cDNA clones were subjected to sequence analysis: 14 clones contained a GCAUG sequence and two clones a GCACG sequence (Figure 3A). The function of this sequence was examined by gel shift experiments (Figure 3B). When the No. 10 RNA probe, which contained the GCAUG sequence, was used, strong binding of zFox-1 was observed (lanes 1–3). Fox-1 bound to the GCACG sequence weakly (lanes 10–12). In contrast, zFox-1 binding was not detectable in the case of mutant RNA probes (lanes 4–9). Fox-1 binding was not detected in the case of the No. 13 probe, which contained neither GCAUG nor GCACG (data not shown). We found that binding efficiency to the GCAUG sequence was dependent on the amount of Fox-1 (Figure 3C). Moreover, binding of Fox-1 to GCAUG was competed out by

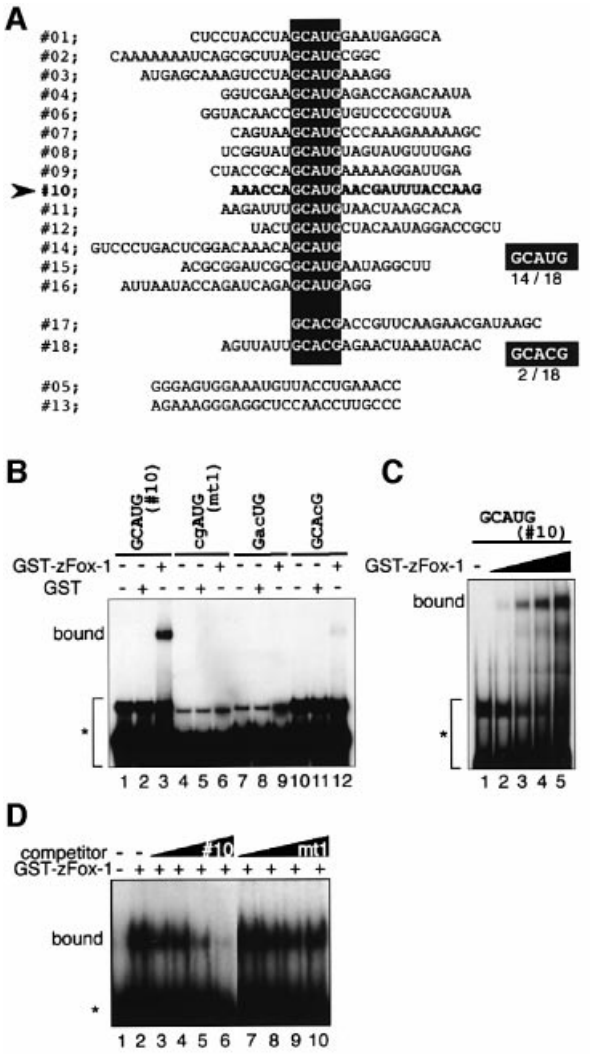

Fig. 3. Fox-1 protein binds specifically to GCAUG *in vitro*. (**A**) In the *in vitro* selection experiment, the sequences of 18 cDNA clones were aligned. The sequences GCAUG and GCACG are boxed. (**B**) Gel shift analyses of Fox-1 protein. No. 10 RNA (lanes 1–3) and mutant RNAs (lanes 4–12) were incubated with GST (lanes 2, 5, 8 and 11) or GST–zFox-1 (lanes 3, 6, 9 and 12). The position of unbound probe is shown by an asterisk. (**C**) Dose-dependent binding of Fox-1 protein to No. 10 RNA. Various amounts of GST–zFox-1 protein were incubated with the No. 10 RNA (0, 250, 500, 750 or 1000 ng, from left to right). (**D**) Competition experiments for GCAUG binding. No. 10 RNA was used as a probe. No.10 (lanes 3–6) or its mutant RNA (cgAUG) (lanes 7–10) was added as competitor (10-, 50-, 250-and 1250-fold excess compared with the ^{32}P-labeled probe).

GCAUG-containing RNA, not by RNA containing the mutant sequence (Figure 3D). These results clearly indicate that Fox-1 binds to GCAUG *in vitro*.

Fox-1 induces muscle-specific exon skipping of F1γ pre-mRNA via GCAUG

Our findings suggested that Fox-1 is involved in the regulation of muscle-specific splicing via GCAUG in vertebrates. Muscle-specific splicing has been well characterized for several mammalian genes, although there are no reports that GCAUG is involved in muscle-specific splicing. As candidates for muscle-specific targets of Fox-1, we focused on two genes, the human mitochondrial ATP synthase γ-subunit gene (F1γ) (Hayakawa *et al*., 2002) and the rat α-actinin gene (Southby *et al*., 1999), because their primary transcripts contain several copies of GCAUG.

In the case of human F1γ, exon 9 is excluded from the splicing product in a muscle-specific manner (Hayakawa *et al*., 2002) (Figure 4A). We found several copies of the GCAUG sequence in intron 8 in the human and mouse F1γ genes. To examine whether Fox-1 controls alternative splicing of F1γ pre-mRNA, we performed transfection experiments with a human F1γ mini-gene. When the mini-gene hF1γL was transfected into mouse fibrosarcoma L929 cells, the predominant splicing product generated was non-muscle mRNA, and only a small amount of muscle-type product lacking exon 9 was detected (Figure 4A, lane 1). In contrast, muscle-type mRNA was produced efficiently in the presence of zebrafish or mouse Fox-1 (Figure 4A, lanes 3 and 6). Essentially the same results were obtained with a mouse F1γ mini-gene (Ichida *et al*., 2000) (data not shown). Furthermore, essentially the same results were obtained in mouse fibroblast NIH-3T3, monkey kidney epithelial CV-1 and mouse myoblast C2C12 cells (data not shown). Thus, Fox-1 can induce muscle-specific exon skipping of mammalian F1γ pre-mRNA irrespective of the cell type.

To examine if the RNA-binding ability of Fox-1 is essential for the splicing regulation, we introduced an amino acid substitution into the RNP1 motif (Burd and Dreyfuss, 1994) of Fox-1. As expected, the mutant protein zFox-1 F190A did not have RNA-binding activity *in vitro* (data not shown). We confirmed the nuclear localization of zFox-1 F190A protein with a Myc tag (data not shown). When the hF1γL mini-gene was co-transfected with zFox-1 F190A, muscle-specific splicing was not induced (Figure 4A, lane 2), indicating that the RNA-binding activity of Fox-1 is indispensable for the splicing regulation.

We next examined whether Fox-1 regulates muscle-specific splicing via binding to GCAUG. We transfected an F1γ mini-gene (hF1γS) that contained only one copy of the GCAUG sequence in intron 8 due to a large deletion in the intronic sequence (Figure 4B). We found that exon 9 skipping was induced by zebrafish or mouse Fox-1 (Figure 4B, lanes 1–3). Next, we introduced base substitutions into the GCAUG sequence of hF1γS. Transfection analysis showed that the muscle-type mRNA from the resultant mini-gene hF1γS-mt was barely detectable even in the presence of Fox-1 (Figure 4B, lanes 4–6). These results indicate that Fox-1 induces muscle-specific splicing via binding to the GCAUG sequence in vertebrates.

In *Drosophila*, binding of Sxl protein to the non-sex-specific acceptor region of *tra* pre-mRNA is sufficient for induction of female-specific splicing (Inoue *et al*., 1990) via interference with an essential splicing factor, U2AF (Valcarcel *et al*., 1993). To examine whether Fox-1 induces muscle-specific exon skipping simply by binding to F1γ pre-mRNA, we expressed truncated zFox-1 proteins, ΔN and ΔC, fused with the SV40 nuclear localization signal (NLS). Both proteins were localized in the nucleus (data not shown). We found that zFox-1ΔC, in which the C-terminal 122 residues were truncated, did not induce muscle-specific splicing of the F1γ pre-mRNA, while zFox-1ΔN, in which the N-terminal 145 residues

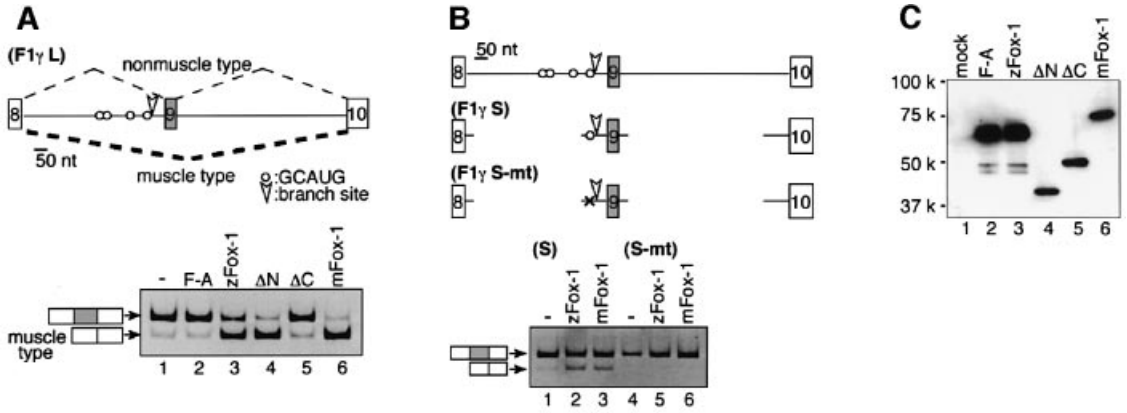

Fig. 4. Fox-1 induces muscle-specific splicing of the human mitochondrial ATP synthase F1γ gene via binding to GCAUG. (**A**) Transfection analyses with the hF1γL mini-gene. Exon 9 is skipped in a muscle-specific manner (Hayakawa *et al*., 2002). The positions of the branch site (arrowhead) and the GCAUG sequences (open circles) are shown in the schematic representation of the mini-gene. The mini-gene was transfected into L929 cells along with pCS2+ vector (Rupp *et al*., 1994) (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1ΔN (lane 4), zFox-1ΔC (lane 5) or mouse Fox-1 (lane 6). The positions of non-muscle and muscle-type splicing products are indicated on the left. (**B**) Transfection analyses of the hF1γS mini-gene containing the Fox-1-binding sequence GCAUG (lanes 1–3) or the mutant sequence cgAUG (lanes 4–6) with pCS2+ MT vector (lanes 1 and 4), zFox-1 (lanes 2 and 5) or mouse Fox-1 (lanes 3 and 6). (**C**) Western blotting of the cell extracts to detect Fox-1 proteins: mock (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1ΔN (lane 4), zFox-1ΔC (lane 5) and mouse Fox-1 (lane 6), expressed from the pCS2+ MT vector using the anti-Myc antibody.

were truncated, induced muscle-specific splicing (Figure 4A, lanes 4 and 5). UV cross-linking experiments showed that the truncated Fox-1 proteins ΔN and ΔC had the same binding specificity as the intact Fox-1 protein *in vitro* (data not shown). These results suggest that the induction of muscle-specific exon skipping of F1γ pre-mRNA by Fox-1 probably involves the association of Fox-1 with some protein(s) via its C-terminal region.

Fox-1 regulates mutually exclusive splicing of α-actinin

It has been reported that the rat α-actinin gene produces two mRNA isoforms in which the upstream non-muscle (NM) exon and the downstream smooth muscle (SM) exon are selected in a mutually exclusive manner (Southby *et al*., 1999) (Figure 5A). Several copies of the GCAUG sequence lie in the introns flanking the NM exon (Southby *et al*., 1999). When the actinin mini-gene (Southby *et al*., 1999) alone was transfected into CV-1 cells, mostly NM-type mRNA was generated, and only a small amount of SM mRNA was detected (Figure 5A, lane 1). When zFox-1 protein was co-expressed, the SM-type mRNA increased, with a concomitant decrease of the NM mRNA (lane 3). In contrast, zFox-1 F190A did not induce the SM-type splicing (lane 2). Essentially the same results were obtained in L929 cells (data not shown). These results indicate that zFox-1 induces SM-specific splicing of α-actinin.

We considered three possibilities by which Fox-1 might regulate mutually exclusive splicing of α-actinin. The first possibility is that Fox-1 simply represses NM splicing, leading to the usage of the SM exon. The second possibility is that Fox-1 promotes the inclusion of the SM exon without repressing NM splicing. The third possibility is that Fox-1 not only represses NM splicing but also promotes SM splicing. To test whether Fox-1 represses NM splicing, we constructed a chimeric mini-gene, EF-NM/14–15, in which a fragment extending from the EF1a exon to the NM exon of the actinin gene was fused with a fragment extending from exon 14 to exon 15 of the chicken δ-crystallin gene (Figure 5C). The crystallin pre-mRNA containing exon 14 to exon 15 is known to be spliced constitutively (Ohno *et al*., 1987). Transfection experiments showed that an mRNA product containing the chimeric exon NM/14 was generated exclusively in the absence of Fox-1 (Figure 5C, lane 1). In contrast, chimera exon skipping was induced by Fox-1 (lane 3). zFox-1 F190A mutant protein did not induce exon skipping (lane 2). These results indicate that NM splicing is repressed by Fox-1. Furthermore, we found that zFox-1ΔC did not induce exon skipping, although zFox-1ΔN did, suggesting that the C-terminal region of zFox-1 plays a role in repressing NM splicing (lanes 4 and 5). Using the original actinin mini-gene, we further examined the effects of truncated Fox-1 proteins (Figure 5A, lanes 4 and 5). When zFox-1ΔC was expressed, SM splicing was not induced (lane 5). Unexpectedly, when zFox-1ΔN was expressed, muscle-specific splicing of actinin pre-mRNA was not induced. Instead, an additional mRNA product lacking both the NM and SM exons was produced (lane 4). We designated this process ‘double exclusion’. It is likely that zFox-1ΔN can induce NM exon skipping, but not SM exon inclusion, resulting in double exclusion. The results suggested that Fox-1 promotes SM exon inclusion. To test this possibility further, we constructed an actinin mini-gene extending from the NM exon to the EF2 exon (NM-SM-EF2). When cells were transfected with the mini-gene alone, exclusion of the SM exon was mainly observed (Figure 5D, lane 1). In contrast, SM exon inclusion was enhanced by Fox-1, although the enhancement was not so efficient (lane 3). zFox-1 F190A did not induce SM exon inclusion (lane 2). Furthermore, zFox-1ΔN did not induce SM exon inclusion, although zFox-1ΔC did (lanes 4 and 5), which was consistent with the results shown in

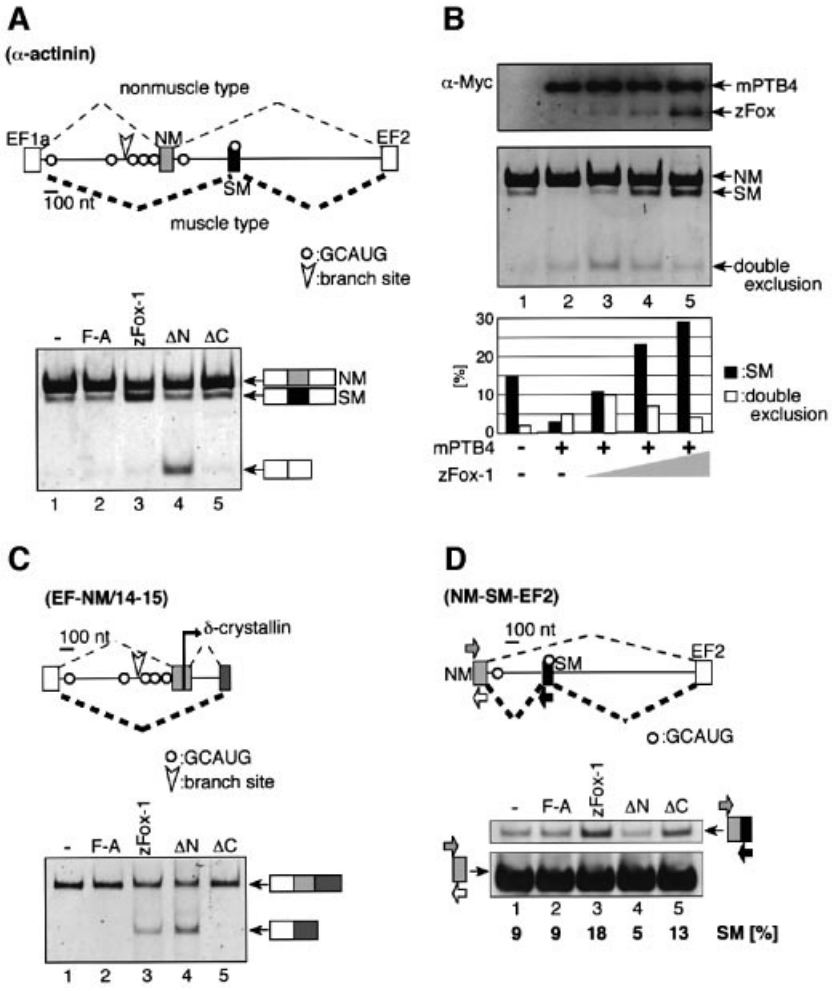

Fig. 5. Muscle-specific splicing of the rat α-actinin gene is induced by Fox-1. (**A**) Transfection analyses of the rat α-actinin mini-gene (Southby *et al*., 1999; Suzuki *et al*., 2002). In the schematic representation, NM and SM represent non-muscle- and smooth muscle-specific exons, respectively. The branch site upstream of the NM exon and the GCAUG sequences are shown. The mini-gene was co-expressed in CV-1 cells along with pCS2+ MT vector (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1ΔN (lane 4) or zFox-1ΔC (lane 5). The position of each mRNA product is indicated on the right. (**B**) Antagonism between the effects of Fox-1 and mouse PTB4. Transfection of the actinin mini-gene with pCS2+ MT vector (lane 1) or mouse PTB4 (lanes 2–5: 1.5 μg of the expression plasmid) and zFox-1 (lanes 3–5: 0.03, 0.09 and 0.27 μg, respectively, of the plasmid). The upper panel shows western blotting of the cell extracts to detect Fox-1 and PTB proteins expressed from the pCS2+ MT vector using the anti-Myc antibody. The splicing products from the actinin mini-gene were analyzed by RT–PCR (middle panel), and the amounts of SM mRNA (black bars) and EF1-EF2 mRNA (white bars) were expressed relative to the total amount of splicing products (lower panel). (**C**) Transfection analyses of the chimera construct, EF-NM/14–15, with pCS2+ MT vector (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1ΔN (lane 4) or zFox-1ΔC (lane 5). The position of each mRNA product is shown schematically on the right. (**D**) Transfection analyses of the NM-SM-EF2 mini-gene with pCS2+ MT vector (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1 ΔN (lane 4) or zFox-1 ΔC (lane 5). The primer sets are shown schematically (arrows). The fraction of SM exon inclusion (percentage) is shown at the bottom of each lane.

Figure 5A. Taking these results together, we conclude that Fox-1 not only represses NM splicing but also activates SM splicing of rat α-actinin.

It has been reported that PTB plays a key role in exclusion of the SM exon of actinin pre-mRNA in non-muscle cells (Southby *et al*., 1999; Wollerton *et al*., 2001). We found that double exclusion was slightly induced by mouse PTB4, with concomitant loss of the SM mRNA (Figure 5B, lanes 1 and 2). In the presence of mouse PTB4, a small amount of Fox-1 induced double exclusion as well as SM splicing (lane 3), suggesting that both the NM and SM exons are repressed in a fraction of actinin pre-mRNA. However, a larger amount of Fox-1 promoted SM splicing, with a concomitant decrease of double exclusion (lanes 4 and 5). These results indicate that Fox-1 antagonizes the repressive effect of PTB to promote muscle-specific splicing of α-actinin.

Fox-1 promotes inclusion of fibronectin EIIIB exon

It is known that the GCAUG sequence plays a pivotal role in the splicing regulation of some genes. In those cases, the GCAUG sequence is essential for exon inclusion. We examined whether Fox-1 regulates the inclusion of the fibronectin EIIIB exon (Huh and Hynes, 1994; Lim and Sharp, 1998). When the fibronectin 7iBi89 mini-gene (Huh and Hynes, 1993, 1994) alone was transfected into CV-1 cells, EIIIB exon inclusion was barely detectable (Figure 6, lane 1). Co-transfection of a plasmid expressing zebrafish or mouse Fox-1 induced a low but significant amount of EIIIB exon inclusion (lanes 3 and 6). The

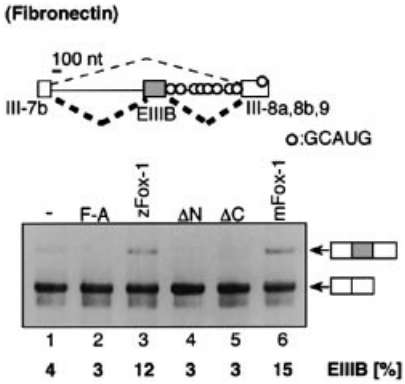

Fig. 6. Inclusion of the rat fibronectin EIIIB exon is promoted by Fox-1. The fibronectin 7iBi89 mini-gene (Huh and Hynes, 1993, 1994) was transfected into CV-1 cells with pCS2+ MT vector (lane 1), zFox-1 F190A (lane 2), zFox-1 (lane 3), zFox-1ΔN (lane 4), zFox-1ΔC (lane 5) or mouse Fox-1 (lane 6). The position of each mRNA product is shown on the right. The fraction of EIIIB exon inclusion (percentage) is shown at the bottom of each lane.

mutant Fox-1 protein F190A did not induce the exon inclusion (lane 2). These results clearly indicated that Fox-1 can induce the inclusion of the fibronectin EIIIB exon. In addition, the truncated Fox-1 proteins ΔN and ΔC did not induce the EIIIB exon inclusion (lanes 4 and 5), suggesting that both the N- and C-terminal portions of Fox-1 protein are important for promoting exon inclusion.

Discussion

Here we showed that Fox-1 regulated muscle-specific splicing of the F1γ and α-actinin genes as well as alternative splicing of the fibronectin EIIIB exon. We demonstrated that Fox-1 binding to GCAUG is essential for splicing regulation, at least in the case of F1γ pre-mRNA. It is also noteworthy that Fox-1 functions as both a negative and positive regulator of alternative splicing.

We found that mouse Fox-1 (Figure 2B) and human A2BP1 (Shibata *et al*., 2000) are expressed in the brain as well as in the heart and muscles. In mammals, neuron-specific splicing of NMHC-B and c-src is regulated by the GCAUG sequence (Kawamoto, 1996; Modafferi and Black, 1997). It has been shown that the hexanucleotide UGCAUG often lies in flanking introns of brain-specific exons and muscle-specific exons (Brudno *et al*., 2001). Therefore, we think it possible that Fox-1 is involved in brain-specific splicing as well as muscle-specific splicing via GCAUG.

Our present data strongly suggest that Fox-1 protein interacts with some other protein(s). Human A2BP1 was identified originally as a protein interacting with ataxin-2 protein in yeast two-hybrid screening: the C-terminal fragment of human A2BP1 is required for strong interaction with ataxin-2 (Shibata *et al*., 2000). The present study showed that the N-terminal portion of zFox-1 is required for positive regulation of splicing, while the C-terminal region is essential for both positive and negative regulation. Thus, it is likely that Fox-1 forms complexes with different proteins to exert these differing activities.

We demonstrated here that Fox-1 induces exon skipping via GCAUG in F1γ pre-mRNA: base substitution in this element in F1γ pre-mRNA resulted in disruption of exon skipping dependent on Fox-1 (Figure 4). This is the first report showing that the GCAUG sequence functions as a splicing silencer. In the case of F1γS pre-mRNA that contained only a single copy of the element, production of muscle-specific mRNA was not so efficient. When three copies of GCAUG were inserted into F1γS-mt, the efficiency of muscle-specific splicing was restored almost to the original level, further supporting the notion that GCAUG functions as a splicing silencer (our unpublished observations).

In the case of α-actinin pre-mRNA, Fox-1 not only repressed NM splicing but also promoted SM splicing (Figure 5). Our data showed that Fox-1 antagonizes the repressive effect of PTB to promote SM splicing, although whether Fox-1 interacts with PTB has not been examined. A recent study showed that one of the CELF/Bruno-like protein family members, Etr-3, induces muscle-specific splicing of cardiac troponin T (cTnT) pre-mRNA, antagonizing the repressive effect of PTB (Charlet *et al*., 2002). In addition, a brain-specific PTB (brPTB) interacts with Nova-1 and antagonizes the ability of Nova-1 to regulate neuron-specific splicing (Polydorides *et al*., 2000). Thus, antagonism between PTB and tissue-specific regulators plays a pivotal role in alternative splicing.

Our previous study revealed that zebrafish CELF/Bruno-like proteins, Bruno-like (Brul) and Etr-3, can induce muscle-specific splicing of rat α-actinin via binding to uridine and purine repeat elements (UREs) at the branch point upstream of the NM exon (Suzuki *et al*., 2002). Co-expression of Fox-1 and Brul resulted in the increased induction of muscle-specific splicing of actinin pre-mRNA, not in a synergistic, but rather in an additive, manner (our unpublished observations). Base substitutions in the URE sequence disrupted the regulation of muscle-specific splicing by Brul, while no significant effect was observed in the case of muscle-specific splicing induced by Fox-1 (our unpublished observations). These results suggest that CELF/Bruno-like proteins and Fox-1 function independently of each other, although it remains possible that CELF/Bruno-like proteins as well as Fox-1 promote muscle-specific splicing of actinin pre-mRNA through some PTB-antagonistic effect.

Fox-1 weakly promoted fibronectin EIIIB exon inclusion (Figure 6). Alternative splicing of the EIIIB exon is regulated in various tissues and stages (Huh and Hynes, 1994; Lim and Sharp, 1998). Another mouse *fox-1*-related gene, *fxh* (or *RBM9*), has been identified as a gene induced by androgens in motor neuron cells (Lieberman *et al*., 2001). Its mRNA is expressed in various tissues such as heart, brain, lung, liver and kidney (Lieberman *et al*., 2001). We found that mouse Fxh/RBM9 protein promotes fibronectin EIIIB exon inclusion, somewhat more strongly than Fox-1, suggesting its role in splicing regulation in various tissues (unpublished observations). We think that there are at least two possibilities for how Fox-1 (or a Fox-1-related protein) promotes fibronectin EIIIB exon inclusion. The first possibility is that Fox-1 directly induces assembly of splicing factors through binding to the GCAUG sequences. The second possibility is that Fox-1 antagonizes the repressive effect of PTB, as is the

case for regulation of SM splicing in actinin pre-mRNA. It has been reported that PTB is involved in the regulation of fibronectin EIIIB exon inclusion (Norton and Hynes, 1993; Norton, 1994).

In *C.elegans*, it has been suggested that *fox-1* regulates sex-specific splicing of *xol-1* pre-mRNA (Skipper *et al*., 1999). Exon 7 is skipped in hermaphrodites, i.e. in the presence of Fox-1 (Meyer, 2000). We found two copies of GCAUG in the intron upstream of exon 7, and several copies of GCACG in the flanking introns. Furthermore, the nematode Fox-1 protein was able to induce muscle-specific exon skipping of the F1γ pre-mRNA in mammalian cells (our unpublished observations). Thus, it is possible that the mechanism of regulation of alternative splicing by Fox-1 proteins is somehow conserved in widely divergent species. We expect that further studies of Fox-1 will provide novel and significant information about the regulation of alternative splicing.

Materials and methods

Plasmids

A zebrafish neurula cDNA library in the ZAPII vector was screened to obtain a *fox-1* cDNA clone (DDBJ/EMBL/GenBank accession No. AB074763). The mouse PTB4 cDNA was cloned by RT–PCR using total RNA from E9.5 whole embryos (DDBJ/EMBL/GenBank accession No. AB074764). The coding sequences of zebrafish Fox-1, mouse Fox-1/A2BP1 (NM_021477) and mouse PTB4 were cloned into pBluescript SK (Stratagene), pGEX-6P-1 (Pharmacia) and pCS2+ MT (Rupp *et al*., 1994) vectors. To construct deletion mutants of Fox-1 (ΔC and ΔN), the *fox-1* cDNA fragments corresponding to nucleotides 1–780 and 463–1146, respectively, were inserted into a pCS2+ MT-derived vector that contained the sequence coding for the SV40 NLS (PKKKRKVKL). Mutagenesis was carried out using a Quikchange site-directed mutagenesis kit (Stratagene). The hF1γL mini-gene includes genomic fragments corresponding to nucleotides 13 648–13 853 and 17 433–18 568 of the human F1γ gene (Matsuda *et al*., 1993) in the pcDEB vector (Ichida *et al*., 2000), while the hF1γS mini-gene (pF1γEx8-9-10; Hayakawa *et al*., 2002) includes genomic fragments corresponding to nucleotides 13 650–13 741, 17 733–17 866 and 18 405–18 572 in the pCMV-SPORT vector (Life Technologies Inc.). A chimeric mini-gene, EF-NM/14–15, was constructed as follows. The genomic fragment of the α-actinin gene corresponding to nucleotides 51–1151 (Southby *et al*., 1999) was amplified by PCR and cloned into pCS2+ (Rupp *et al*., 1994) (pEF-NM). The *Hin*dIII–*Xba*I fragment of pSP14–15 (Sawa *et al*., 1988) was inserted into pEF-NM. To construct the NM-SM-EF2 mini-gene, the genomic fragment of the α-actinin gene corresponding to nucleotides 1132–2923 (Southby *et al*., 1999) was amplified by PCR and cloned into pCS2+ (Rupp *et al*., 1994).

In situ hybridization and northern blotting

Whole-mount *in situ* hybridization of zebrafish embryos was performed essentially as described previously (Maegawa *et al*., 1999). The RNA probe was prepared from *fox-1* cDNA in pBluescript SK (Stratagene) using T3 RNA polymerase. Northern blot analysis of mouse Fox-1 was performed using MessageMap Northern Blot (Stratagene) as described previously (Maegawa *et al*., 1999).

SELEX and RNA-binding experiments

Recombinant GST fusion proteins were induced in *Escherichia coli* BL21(DE3) or DH5, and purified on glutathione–agarose beads (Pharmacia). *In vitro* selection experiments were carried out essentially as described previously (Abe *et al*., 1996; Suzuki *et al*., 2002). The starting RNA source was synthesized *in vitro* from a mixture of DNA templates containing a T7 promoter and a randomized 25 nucleotide region. A total of five rounds of selection and amplification were performed. The final cDNA products were inserted into pSP65 (Promega). Preparation of RNA probes and *in vitro* binding experiments were performed essentially as described previously (Ohno *et al*., 1990; Suzuki *et al*., 2002).

Transfection experiments

L929 and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection was performed by the calcium phosphate DNA precipitation method as described previously (Suzuki *et al*., 2002). The Myc fusion proteins expressed in transformant cells were examined by western blotting and immunofluorescence detection using anti-Myc antibody (cMyc 9E10; Santa Cruz Biotechnology). To analyze splicing products, RT–PCR was performed using the following oligonucleotides. For hF1γ(L), F1-2903 (GTCATCACAAAAGAGTTGATTG) and F1-2389 (CACTGCATTC-TAGTTGTGGTTTGT); for hF1γ (S), F1-2903 and the T7 primer (Stratagene); and for fibronectin, 7iBi89-S (TTCGAATTCATC-AGAGTTCCTGCACT) and hGHpolyA-AS (CTGCTCGAGACTGG-AGTGGCAACTTC) were used. For NM-SM-EF2, NM-S (CGG-CTCGAGGATCACTCCGGCACGTTGGG) and SM-AS (CCACTC-GAGAACCCATGGAGATAAGGCAG) were used to analyze SM exon inclusion, while NM-S and NM-AS (GGGTCGTTGCCAATATC) were used to detect total splicing products. For α-actinin except NM-SM-EF2, ACTs121 and ACTas2873 were used (Suzuki *et al*., 2002). The RT–PCR products were electrophoresed in 5% native polyacrylamide gels or 2% agarose gels. After staining with Sybr green I (Molecular Probe), the products were analyzed using an FM-BIO II bioimager (Hitachi). Each transfection experiment was performed more than three times to confirm the reproducibility.

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