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Characterization of U2AF^{26}, a Splicing Factor Related to U2AF^{35}

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The essential splicing factor U2AF (U2 auxiliary factor) is a heterodimer composed of 65-kDa (U2AF^{65}) and 35-kDa (U2AF^{35}) subunits. U2AF^{35} has multiple functions in pre-mRNA splicing. First, U2AF^{35} has been shown to function by directly interacting with the AG at the 3′ splice site. Second, U2AF^{35} is thought to play a role in the recruitment of U2AF^{65} by serine-arginine-rich (SR) proteins in enhancer-dependent splicing. It has been proposed that the physical interaction between the arginine-serine-rich (RS) domain of U2AF^{35} and SR proteins is important for this activity. However, other data suggest that this may not be the case. Here, we report the identification of a mammalian gene that encodes a 26-kDa protein bearing strong sequence similarity to U2AF^{35}, designated U2AF^{26}. The N-terminal 187 amino acids of U2AF^{35} and U2AF^{26} are nearly identical. However, the C-terminal domain of U2AF^{26} lacks many characteristics of the U2AF^{35} RS domain and, therefore, might be incapable of interacting with SR proteins. We show that U2AF^{26} can associate with U2AF^{65} and can functionally substitute for U2AF^{35} in both constitutive and enhancer-dependent splicing, demonstrating that the RS domain of the small U2AF subunit is not required for splicing enhancer function. Finally, we show that U2AF^{26} functions by enhancing the binding of U2AF^{65} to weak 3′ splice sites. These studies identify U2AF^{26} as a mammalian splicing factor and demonstrate that distinct U2AF complexes can participate in pre-mRNA splicing. Based on its sequence and functional similarity to U2AF^{35}, U2AF^{26} may play a role in regulating alternative splicing.

Introns are removed from nascent transcripts by the process of pre-mRNA splicing. This reaction is catalyzed by the spliceosome, a large, multicomponent RNA-protein machine, which assembles on each intron targeted for removal (4). Spliceosome assembly is directed by conserved sequences within introns and at intron-exon boundaries. In mammals, these signals consist of a single sequence at the 5′ splice site (AG/GURAG) and a set of three sequences at the 3′ splice site (4). The latter consists of the branch point, a pyrimidine tract of ~20 nucleotides (nt), and the sequence YAG that defines the end of the 3′ splice site. The first step in spliceosome assembly, the formation of the E complex, involves the binding of U1 snRNP to the 5′ splice site, SF1/mBBP to the branch point, and U2 snRNP auxiliary factor (U2AF) to the pyrimidine tract and YAG (23, 26). In addition, U2 snRNP is associated with the spliceosome at this point, but not stably bound to the pre-mRNA (6). Subsequently, U2 snRNP replaces SF1 at the branch point to form the A complex (26). Understanding the details of these early events in spliceosome assembly is important because alternative splicing is frequently regulated during these steps (31).

U2AF is a heterodimeric complex conserved in all metazoan species. In mammals, this complex consists of 65-kDa (U2AF^{65}) and 35-kDa (U2AF^{35}) subunits (42). U2AF^{65} contains an N-terminal arginine-serine-rich (RS) domain and three RNA recognition motifs (RRM) (43). U2AF^{65} is an essential splicing factor in vitro and is required for viability in *Drosophila melanogaster* (15), *Caenorhabditis elegans* (20) and *Schizosaccharomyces pombe* (25). U2AF^{65} interacts directly with the pyrimidine tract and is involved in stabilizing the interaction of U2 snRNP with the branch point (8, 35). This activity requires the RS domain, which is thought to assist in the formation of the U2 snRNP-pre-mRNA duplex (35), and the third RRM, which interacts with SAP 155, a component of U2 snRNP (8).

The role of U2AF^{35} in pre-mRNA splicing has been more controversial than that of U2AF^{65}. U2AF^{35} contains two zinc fingers and a noncanonical RRM (1) in the N terminus of the protein and an RS domain and a glycine tract at the C terminus (44). The precise role of U2AF^{35} in pre-mRNA splicing has been unclear because some studies have shown that U2AF^{35} is dispensable for splicing in vitro (14, 41), while other studies indicate that U2AF^{35} is an essential splicing factor in vitro (12, 49). Nonetheless, the small U2AF subunit is required for viability in *Drosophila* (15) and *C. elegans* (48), as is its interaction with U2AF^{65} (29). One function of U2AF^{35} in pre-mRNA splicing was recently clarified when three groups demonstrated that U2AF^{35} associates with the AG at the 3′ splice site (21, 40, 47).

In addition to constitutive splicing, U2AF^{35} appears to play an important role in regulated splicing. The removal of many introns requires *cis*-acting sequences present in the downstream exon called exonic splicing enhancers (ESEs) (2, 10). Many ESEs are recognized by serine-arginine-rich (SR) proteins—a conserved family of essential splicing factors (10). Several studies have shown that enhancer-bound SR proteins function by recruiting U2AF^{65} to weak pyrimidine tracts (3, 11, 36, 49). It has been proposed that U2AF recruitment involves protein interactions between enhancer-bound SR proteins and

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the RS domain of U2AF^{35} (39, 49). However, other studies indicate that ESEs may function through a mechanism that requires neither U2AF recruitment (14, 19) nor U2AF^{35} (14). Interestingly, the small U2AF subunits from *C. elegans* (48) and *S. pombe* (37) do not contain RS domains. Moreover, transgenic *Drosophila* expressing a version of dU2AF^{38} lacking its RS domain as the sole source of dU2AF^{38} is not only viable but properly regulates the alternative splicing of the enhancer-dependent *doublesex* pre-mRNA (28). Thus, at least in nonvertebrate systems, the RS domain is not an essential feature of the small U2AF subunit.

Here, we report the identification and characterization of a mammalian gene, *U2AF^{26}*, capable of encoding a protein product very similar to U2AF^{35}. The N-terminal portion of the U2AF^{26} polypeptide, which contains two zinc fingers, a noncanonical RRM, and a U2AF^{65} interaction domain, is 89% identical to U2AF^{35}. However, the C-terminal domain of U2AF^{26} is quite different from the C-terminal domain of U2AF^{35}—most of the RS dipeptides, as well as the entire glycine tract present in U2AF^{35}, are absent in U2AF^{26}. We find that the relative levels of U2AF^{35} and U2AF^{26} mRNAs vary in different mouse tissues. U2AF^{26} is a nuclear protein that localizes to speckles and can physically associate with U2AF^{65}. Biochemical experiments demonstrate that U2AF^{26} functions as a pre-mRNA splicing factor and can functionally substitute for U2AF^{35} in constitutive splicing in vitro. Interestingly, U2AF^{26} can also replace U2AF^{35} in enhancer-dependent splicing, demonstrating that although a small U2AF subunit is necessary for efficient enhancer-dependent splicing, its RS domain is not. Finally, we show that U2AF^{26} enhances the binding of U2AF^{65} to weak 3′ splice sites. These studies identify U2AF^{26} as a mammalian splicing factor that may participate in the regulation of alternative splicing.

MATERIALS AND METHODS

Cloning of *U2AF^{26}*. We identified a cDNA fragment corresponding to the N terminus of U2AF^{26} in a screen for genes regulated by the transcription factor NPAS2 (45). This fragment was used to screen an adult mouse brain Uni-ZAP XR cDNA library (Stratagene). Two overlapping partial cDNA clones were used to assemble a full-length *U2AF^{26}* cDNA. The assembled cDNA sequence was confirmed by reverse transcription-PCR experiments from mouse brain RNA as well as multiple expressed sequence tags (ESTs) in GenBank. A mouse *U2AF^{35}* cDNA was cloned from total brain RNA by reverse transcription-PCR. Using the mouse *U2AF^{26}* cDNA as a probe, we screened a mouse 129/SvEV Tac f BR genomic library (Strategene) for the *U2AF^{26}* gene. Using overlapping genomic clones, we were able to assemble a contig containing the complete *U2AF^{26}* gene.

Northern blotting. Total RNA was isolated from dissected mouse brain, cerebellum, colon, eyes, heart, kidney, liver, lung, skeletal muscle, small intestine, spleen, testis, uterus, and white adipose tissue using RNA Stat-60 (Tel-Test). Poly(A)$^+$ RNA was isolated by passing the RNA samples twice through oligo(dT) cellulose columns (Gibco). A total of 5 μg of RNA was run on a 1.2% formaldehyde gel, transferred to a Nytran supercharge membrane, and hybridized with ^{32}P-labeled DNA probes. The *U2AF^{26}* transcripts were detected with a full-length cDNA probe, while the *U2AF^{35}* transcripts were detected with a probe corresponding to the last 476 bp of the mouse cDNA. The blots were also probed with a cyclophilin probe as an internal control for RNA loading. The blots were developed and quantitated with a Fuji BAS1500 phosphorimager. After normalization to the cyclophilin control, the U2AF^{35} and U2AF^{26} signals in the lung tissue, which was the tissue expressing the median value of each mRNA, were set to a value of 1.0. The U2AF^{35} and U2AF^{26} signals in the remainder of the tissues were normalized to the signal in lung tissue, and these values were used to calculate the ratio of U2AF^{26} to U2AF^{35}.

Indirect immunofluorescence. HeLa cells were transfected with 1 μg of pcDNA-mU2AF^{26}/V5, which encoded murine U2AF^{26} (mU2AF^{26}) containing a C-terminal V5 epitope tag, using Fugene6 (Boehringer Mannheim). In addition, cells were transfected with control plasmids expressing either human U2AF^{35} (hU2AF^{35}) or human SF2/ASF containing C-terminal V5 epitope tags. Twenty-four hours after transfection, the cells were fixed in formaldehyde and probed with anti-V5 antibody (Invitrogen). The cells were then washed with 1× phosphate-buffered saline and probed with an anti-mouse immunoglobulin G-fluorescein isothiocyanate secondary antibody (Jackson ImmunoResearch Laboratories). The cells were washed, stained with DAPI (4′,6′-diamidino-2-phenylindole), and visualized with a Zeiss Axioplan 2 microscope, and the images were acquired with a SenSys-cooled charge-coupled device camera using OpenLab software (Improvision).

Expression and purification of recombinant proteins. His$_6$-mU2AF^{26} and His$_6$-hU2AF^{35} were cloned into the baculovirus expression vector, pFastBac-1 (Gibco). The N-terminal His$_6$ tag was added by PCR. To construct a virus encoding GST-hU2AF^{65}, hU2AF^{65} was first cloned into pGEXT-4T (Amersham-Pharmacia), amplified by PCR to isolate the fragment encoding GST-U2AF^{65}, and subsequently cloned into pFastBac-1. Baculoviruses encoding these proteins were produced as described by the manufacturer (Gibco). The U2AF^{65}-U2AF^{26} and U2AF^{65}-U2AF^{35} heterodimers were produced by coinfecting the Sf9 cells with the respective viruses, whereas U2AF^{65} was produced by infecting Sf9 cells with a single virus. At 48 h after infection, the Sf9 cells were centrifuged at 500 × *g* for 10 min and the pellet was resuspended in lysis buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10-μg/ml leupeptin). The suspension was sonicated for 1 min and centrifuged at 18,000 rpm in a JA-20 rotor (Beckman) for 25 min. The supernatant was then incubated for 25 min at 4°C with Ni-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen) equilibrated with lysis buffer. After incubation, the beads were loaded onto a column, washed with lysis buffer, and eluted with lysis buffer containing 300 mM imidazole. The eluate was then incubated with equilibrated glutathione-Sepharose 4B (Amersham Pharmacia) for 25 min and then loaded onto a column. The beads were washed with lysis buffer and eluted with lysis buffer containing 50 mM glutathione. U2AF^{65} was purified on glutathione-Sepharose only. The purified proteins were dialyzed overnight in buffer D (100 mM KCl, 20% glycerol, 20 mM HEPES [pH 7.9], 0.2 mM EDTA, 0.1 mM dithiothreitol).

RNAs. Plasmids encoding the β-globin and *dsx*-avian sarcoma leukosis virus (ASLV) pre-mRNAs were described previously (27, 32). Templates encoding the RNAs used for the gel shift experiments were generated by PCR from the *dsx* or β-globin templates. ^{32}P-labeled RNAs were synthesized with either SP6 or T7 RNA polymerase.

In vitro-splicing assays. HeLa cell nuclear extract was depleted of U2AF^{65} and U2AF^{35} by oligo(dT) chromatography as described previously (11). The depleted extract used in these experiments is the same one used in the experiments reported in reference 11. As shown in reference 11, Western blot analysis suggests that >95% of U2AF^{65} and U2AF^{35} was depleted from this extract. Splicing assays were carried out using 30% U2AF-depleted extract as previously described (11).

U2AF binding assays. The recombinant U2AF preparations were incubated with 5 fmol of ^{32}P-labeled RNAs in reaction mixtures containing 800 ng of BSA, 250 ng of tRNA, 1.3% polyvinylalcohol, 1 mM dithiothreitol, and 4 U of RNasin (Promega) in a 10-μl volume for 15 min at 30°C. The reaction mixtures were transferred to ice, heparin was added to 0.05 mg/ml, and the mixtures were resolved by electrophoresis on 4% 80:1 polyacrylamide gels at 13 V/cm for 3 h at 4°C. The gels were quantitated with a Molecular Dynamics Storm PhosphorImager, and the percentage of RNA bound at each protein concentration was calculated. K_d values were calculated for U2AF^{65} by fitting the data to a single binding isotherm. K_d values for the U2AF heterodimers were calculated by fitting the data to a cooperative binding isotherm where $Y = (K_1[P] + K_1K_2K_C[P]^2)/\{1 + (K_1 + K_2)[P] + K_1K_2K_C[P]^2\}$, where K_1 and K_2 are the estimated equilibrium association constants for U2AF^{65} and U2AF^{35} or U2AF^{26}, Y is the fraction of RNA bound, P is the concentration of protein used, and K_C is the cooperativity factor (13, 38).

RESULTS

Cloning of U2AF^{26} and its similarity to U2AF^{35}. A cDNA fragment encoding a protein similar to U2AF^{35} was identified in a screen for genes regulated by the transcription factor NPAS2 (45). Using this cDNA fragment as a probe, two overlapping partial cDNAs were obtained from a mouse cDNA library and were used to assemble a full-length cDNA. This cDNA encodes a protein specifying a predicted molecular

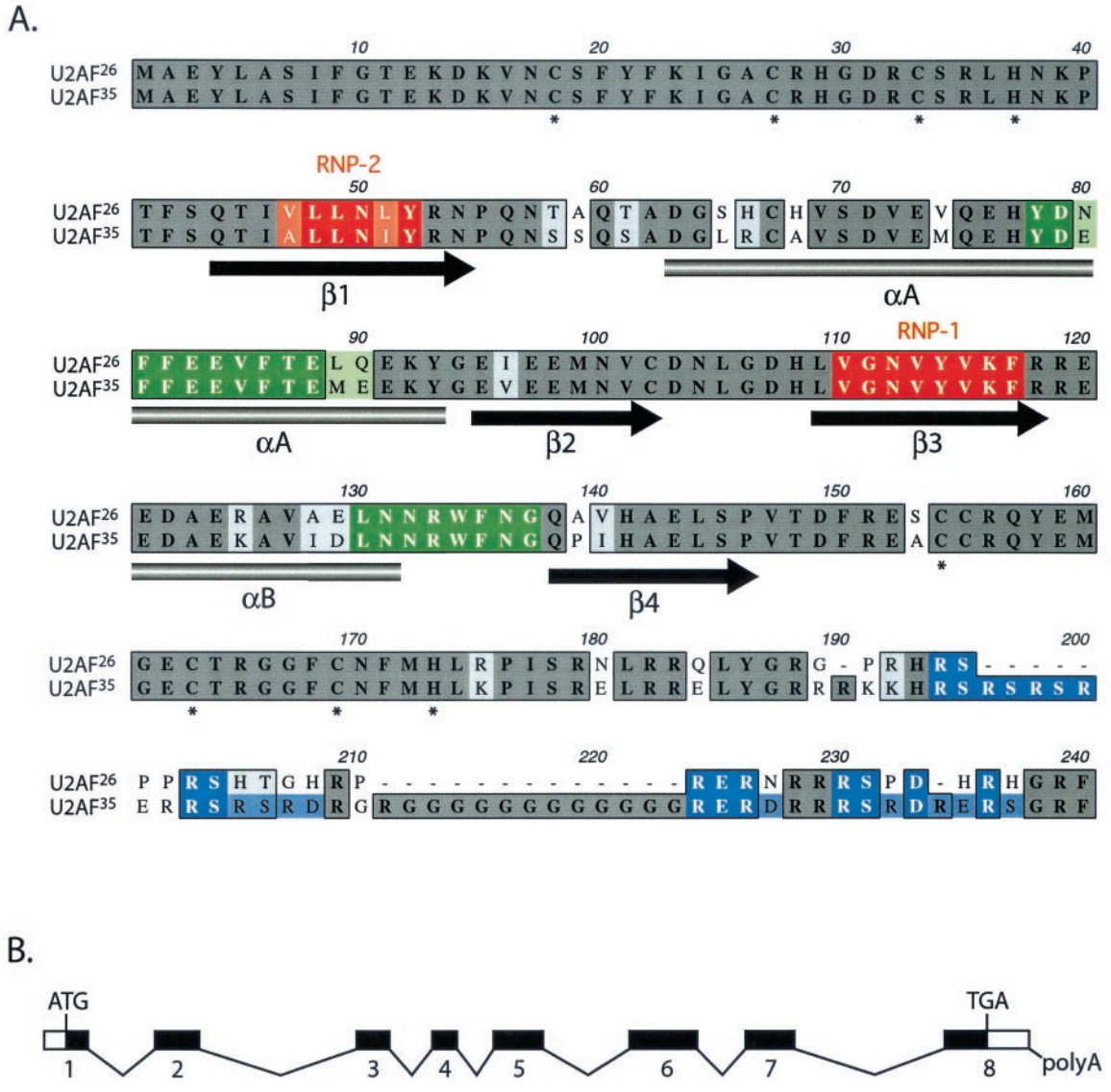

FIG. 1. Sequence comparison of mU2AF^{26} and U2AF^{35} proteins and organization of the *U2AF^{26}* gene. (A) U2AF^{35} and U2AF^{26} share 89% sequence identity within the first 187 residues of the proteins. The dark grey boxes indicate identical amino acids, and the light grey boxes indicate conserved amino acids. The location of the two zinc finger motifs are indicated with asterisks. The RNP1 and RNP2 motifs of the noncanonical RRM are highlighted in red. The amino acids involved in directly contacting U2AF^{65} are highlighted in green. In addition, RS, RE, or RD dipeptides in the C terminus are highlighted in blue. (B) Organization of the mouse *U2AF^{26}* gene.

weight of 26 kDa that is 76% identical to mU2AF^{35}. We therefore designate this protein U2AF^{26}.

U2AF^{35} and U2AF^{26} share 89% primary amino acid sequence identity within the N-terminal 187 residues of the proteins (Fig. 1A). The N-terminal domain contains two zinc fingers that are identical between the two proteins, as well as a noncanonical RRM (1, 17). The RNP-1 portion of the RRM is identical between U2AF^{35} and U2AF^{26} (Fig. 1A). However, the RNP-2 motif of U2AF^{26} contains two amino acid substitutions with respect to U2AF^{35}—a change from Ala to Val at position 47, and a change from Ile to Leu at position 51. The high degree of conservation of the noncanonical RRM suggests that U2AF^{26}, like U2AF^{35}, has the ability to bind RNA (21, 40, 47). In addition, the majority of the residues in U2AF^{35} that directly interact with U2AF^{65} (17, 44) are conserved in U2AF^{26}. For example, the crystal structure of the U2AF^{35}-U2AF^{65} heterodimer (17) revealed that Glu80, Phe81, Glu84, Glu88, Arg133, and Phe135 of U2AF^{35} are in intimate contact with U2AF^{65}. Of these residues, only Glu80 is different in U2AF^{26} (changed to an Asn). Most importantly, Trp134, perhaps the most critical residue involved in the U2AF^{65}-U2AF^{35} interaction (17), is conserved in U2AF^{26}. This suggests that U2AF^{26} most likely can physically interact with U2AF^{65}.

In contrast to the highly conserved N terminus, the C terminus of U2AF^{26} differs markedly from that of U2AF^{35}. The U2AF^{35} C-terminal domain contains an RS domain and a glycine tract (44). U2AF^{35} orthologues have been cloned from *Drosophila* (30), *C. elegans* (48), and *S. pombe* (37). The glycine

tract present in U2AF^{35} is conserved in both the *Drosophila* and *C. elegans* U2AF^{35} orthologues, dU2AF^{38} and CeU2AF^{35}, but not the *S. pombe* orthologue, SpU2AF^{23}. In addition, neither CeU2AF^{35} nor SpU2AF^{23} contain an RS domain, whereas dU2AF^{38} does. The U2AF^{35} RS domain contains seven RS, three arginine-glutamate (RE), and three arginine-aspartate (RD) dipeptides which are all common features of RS domains contained in other proteins (10). By contrast, the C-terminal domain of U2AF^{26} completely lacks a glycine tract, is fairly rich in proline, and contains only three RS, one RE, and no RD dipeptides. Moreover, none of the RS dipeptides in U2AF^{26} are adjacent to one another. The RS domain of U2AF^{35} has been shown to function as a nuclear localization signal (7) and is thought to participate in protein interactions with SR proteins (39). The sequence of the C-terminal domain of U2AF^{26} suggests that both of these activities are altered in U2AF^{26}.

Cloning of the *U2AF^{26}* gene. To compare the genomic organization of the *U2AF^{26}* gene with that of *U2AF^{35}*, we cloned and sequenced the gene encoding U2AF^{26} from a mouse genomic DNA library. The *U2AF^{26}* gene is small, spanning only 2,059 bp (Fig. 1B). Experiments with 5′ rapid amplification of cDNA ends suggest that transcription initiates approximately 50 bp upstream of the start codon. A poly(A) consensus signal resides 114 bp downstream of the stop codon. The gene contains eight exons that when spliced together generate an mRNA of approximately 900 nt. The exon-intron boundaries of the *U2AF^{26}* gene are identical to those in the human *U2AF^{35}* gene located on chromosome 21 (18).

U2AF^{26} is differentially expressed. To examine the expression of U2AF^{26} in comparison to U2AF^{35}, we performed Northern blot analyses of poly(A)$^+$ RNA isolated from different mouse tissues (Fig. 2A). Due to the fact that we could not correct for hybridization efficiency of the probes, we have expressed the results as a ratio of U2AF^{26} to U2AF^{35} signals in each tissue, which was calculated after normalization to the cyclophilin control (see Materials and Methods). These experiments clearly demonstrate that the relative level of U2AF^{26} and U2AF^{35} mRNAs varies in different tissues (Fig. 2B). For example, the ratio of U2AF^{26} to U2AF^{35} is highest in brain (~3) and lowest in liver (~0.5). Thus, U2AF^{26} is differentially expressed in various mouse tissues.

Subcellular localization of U2AF^{26}. To determine the subcellular localization of U2AF^{26}, HeLa cells were transfected with an expression vector encoding U2AF^{26} containing a C-terminal V5 epitope tag. As a control, HeLa cells were transfected with expression vectors encoding U2AF^{35} or the SR protein SF2/ASF, each containing a C-terminal V5 epitope tag. Indirect immunofluorescence revealed that, as expected, U2AF^{35} (7) and SF2/ASF (5) were exclusively localized to the nucleus (Fig. 3). These two proteins were diffusely localized throughout the entire nucleus but were excluded from the nucleolus and were concentrated in a number of subnuclear speckles, a feature common to many splicing factors (22). Likewise, U2AF^{26} was observed to be localized exclusively to the nucleus and associated with nuclear speckles (Fig. 3). These results demonstrate that U2AF^{26}, like U2AF^{35}, is a nuclear protein concentrated in speckles and thus is properly localized to function as a pre-mRNA splicing factor.

Expression and purification of U2AF^{65}-U2AF^{26}. To examine whether U2AF^{26} might function as a pre-mRNA splicing factor, we first needed to express and purify the protein. Attempts to purify recombinant U2AF^{26} alone from *Escherichia coli* or Sf9 cells yielded only insoluble protein. We therefore coexpressed His-tagged U2AF^{26} and glutathione *S*-transferase (GST)-tagged U2AF^{65} in Sf9 cells using baculovirus expression vectors. In parallel, the conventional GST-U2AF^{65}–His-U2AF^{35} heterodimer, as well as GST-U2AF^{65} alone, was expressed and purified. The U2AF heterodimers were purified by a two-step procedure. First, the lysate was passed over a Ni-NTA column to purify His-tagged U2AF^{26} or U2AF^{35}. Secondly, the eluate from the Ni-NTA column was passed over a GST-agarose column to purify the GST-U2AF^{65}. The eluate from the GST-agarose column was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. As expected, U2AF^{65} and U2AF^{35} copurified by this method in a 1:1 stoichiometry (Fig. 4, lane 2). We found that U2AF^{65} and U2AF^{26} also copurified over these two columns in a stoichiometry of approximately 1:1 (Fig. 4, lane 3). Gel filtration analysis of the purified recombinant proteins indicated that U2AF^{26} and U2AF^{65} are present in a complex (data not shown). These results demonstrate that U2AF^{26} can physically interact with U2AF^{65}, suggesting that U2AF^{26} has the ability to modify the activity of U2AF^{65} in pre-mRNA splicing.

U2AF^{26} is a pre-mRNA splicing factor that can functionally replace U2AF^{35} in vitro. Given the similarities between U2AF^{26} and U2AF^{35}, we were interested in testing whether U2AF^{26} could function as a pre-mRNA splicing factor and, if so, comparing its activity to that of U2AF^{35}. We therefore compared the ability of U2AF^{65}, U2AF^{65}-U2AF^{26}, or U2AF^{65}-U2AF^{35} to restore splicing to HeLa cell nuclear extracts depleted of endogenous U2AF^{65} and U2AF^{35} (ΔNE). Previous work had shown that U2AF^{35} enhances the ability of U2AF^{65} to restore splicing in ΔNE (11, 12, 49). We first tested whether U2AF^{26} could function in constitutive splicing. As shown in Fig. 5A, U2AF^{65} alone weakly restores the splicing of β-globin pre-mRNA in ΔNE (Fig. 5A, lane 3). However, both U2AF^{65}-U2AF^{35} and U2AF^{65}-U2AF^{26} were approximately twofold more effective in restoring splicing of this substrate than U2AF^{65} alone (lanes 4 and 5). Similar results were observed for the adenovirus major late pre-mRNA (data not shown). These observations indicate that U2AF^{26} can function as a pre-mRNA splicing factor and that it can functionally substitute for U2AF^{35} in constitutive splicing.

We next tested whether U2AF^{26} could function in enhancer-dependent splicing. For this purpose we used the *dsx*-ASLV pre-mRNA (32). This RNA contains the enhancer-dependent *Drosophila doublesex* intron and the ESE from ASLV (16) positioned 100 nt downstream of the 3′ splice site. The ASLV ESE is recognized by SR proteins present in the HeLa cell nuclear extract that, in turn, activate splicing by recruiting U2AF to the *dsx* 3′ splice site (49). U2AF^{65} alone weakly restored splicing of this pre-mRNA in ΔNE (Fig. 5B, lane 3). By contrast, both U2AF^{65}-U2AF^{35} and U2AF^{65}-U2AF^{26} were five- to sixfold more effective in restoring splicing of the *dsx*-ASLV pre-mRNA in ΔNE than U2AF^{65} alone (lanes 4 and 5). Thus, U2AF^{26} can functionally substitute for U2AF^{35} in both constitutive splicing and enhancer-dependent splicing. These

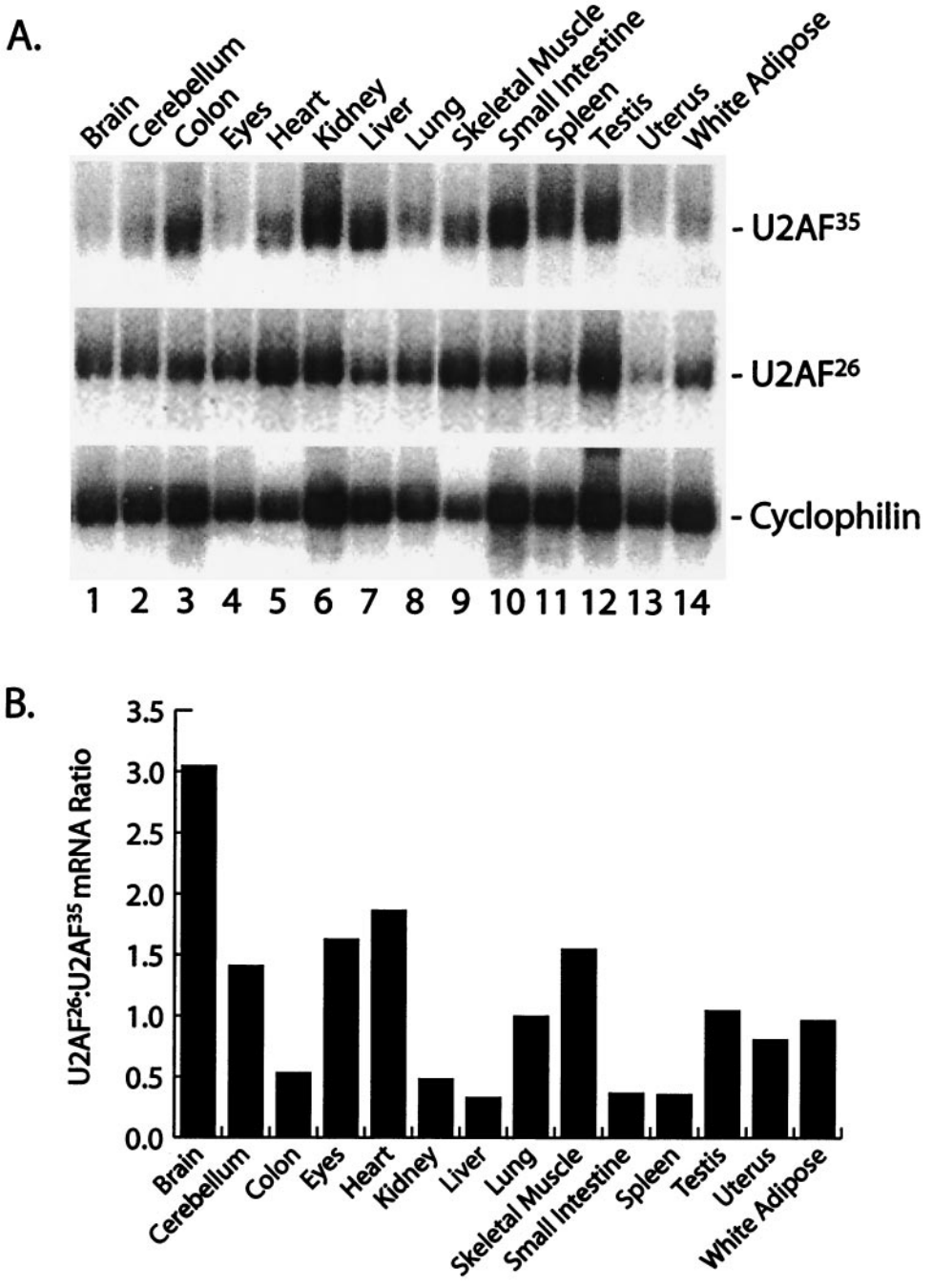

FIG. 2. Comparison of the expression patterns of U2AF^{26} and U2AF^{35} in mouse tissues. Northern blots containing poly(A)$^+$ RNA isolated from the indicated mouse tissues was hybridized with probes specific to the C-terminal domain of mU2AF^{35} (top), full-length mU2AF^{26} (middle), or a cyclophilin control (bottom). (B) The plot depicts the ratio of U2AF^{26} to U2AF^{35} signals in each tissue, which was calculated after normalization to the cyclophilin control as described in Materials and Methods.

results demonstrate that an RS domain in the small U2AF subunit is not required for enhancer-dependent splicing.

U2AF^{26} enhances the binding of U2AF^{65} to weak 3′ splice sites. Our data demonstrate that U2AF^{26} can functionally substitute for U2AF^{35} in both constitutive and enhancer-dependent splicing. We next wanted to determine the basis for the activity of U2AF^{26}. Given that U2AF^{26} appeared to be functionally similar to U2AF^{35}, we reasoned that the activity was most likely due to the sequences within the conserved N terminus rather than the divergent C terminus. Given the known function of U2AF^{35} (21, 40, 47), we thought U2AF^{26} might act by stabilizing the binding of U2AF^{65} on weak 3′ splice sites and that this activity might involve an interaction between U2AF^{26} and the AG at the 3′ splice site. To test this, we performed binding assays using the purified U2AF preparations and RNAs containing various 3′ splice site sequences. As expected,

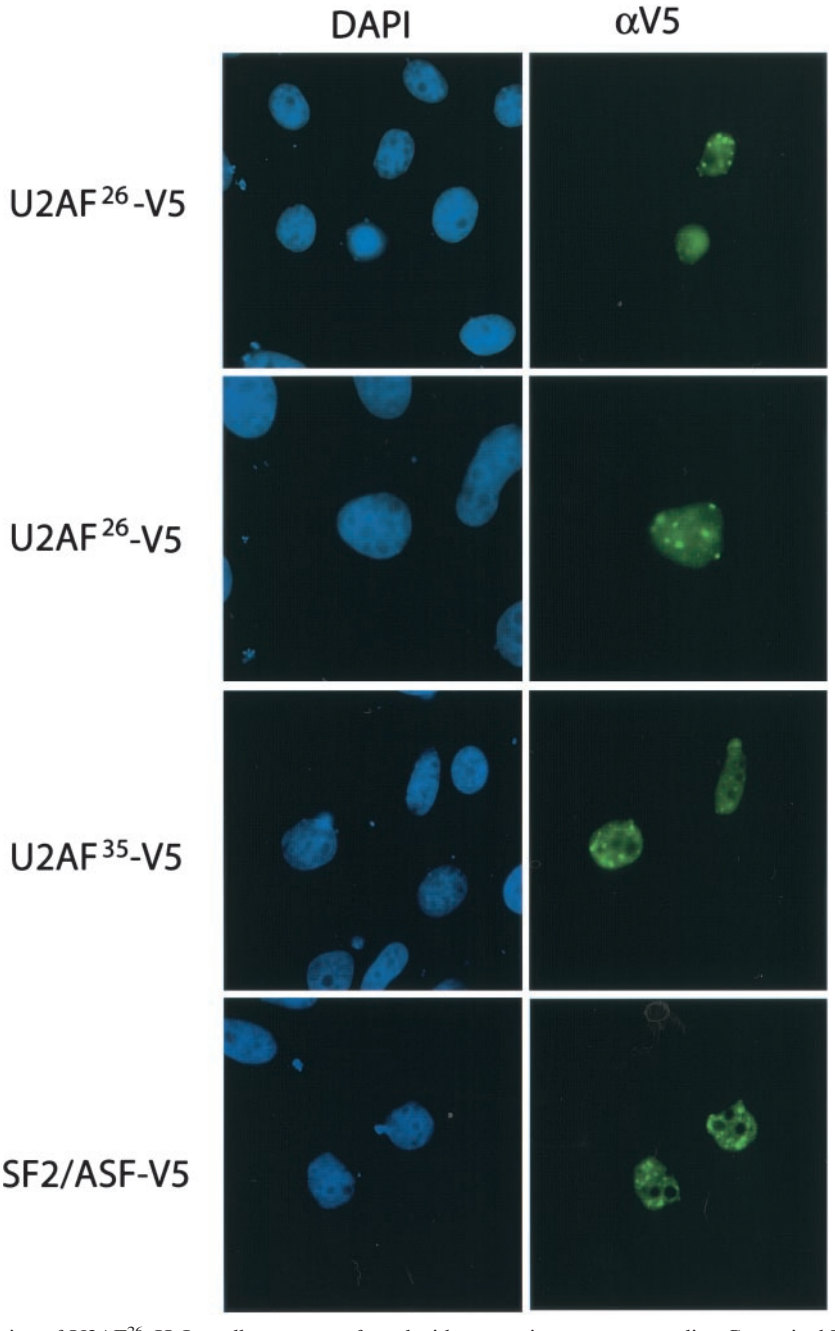

FIG. 3. Immunolocalization of U2AF^{26}. HeLa cells were transfected with expression vectors encoding C-terminal V5-epitope tagged U2AF^{26} (top two rows), U2AF^{35} (third row), or SF2/ASF (bottom row). The cells were fixed and probed with an anti-V5 primary antibody and a fluorescein isothiocyanate-tagged secondary antibody (right). In addition, the cells were stained with DAPI to visualize the nucleus (left). Two separate fields of U2AF^{26} are shown (first and second rows). Speckles cannot be observed in the lower U2AF^{26}-positive cell in the top row because it is out of the plane of focus.

the U2AF^{65}-U2AF^{35} heterodimer bound much more efficiently to a 100-nt RNA containing the weak *dsx* 3′ splice site (K_d = 65 nM) than did U2AF^{65} alone (K_d = 2,000 nM) (Fig. 6A). Surprisingly, the affinity of the U2AF^{65}-U2AF^{26} heterodimer for the *dsx* 3′ splice site (K_d = 60 nM) was nearly identical to that of the U2AF^{65}-U2AF^{35} heterodimer (Fig. 6A). Thus, both U2AF^{35} and U2AF^{26} enhance the affinity of U2AF for the *dsx* 3′ splice site by greater than 30-fold. However, the impact of U2AF^{35} or U2AF^{26} on U2AF binding was substantially diminished on a 100-nt RNA containing the pyrimidine tract from the β-globin pre-mRNA (Fig. 6B) or an RNA containing the consensus U2AF^{65} binding site (data not shown). In the case of the β-globin pyrimidine tract, the difference in affinity of the U2AF heterodimers (U2AF^{65}-U2AF^{35}, 120 nM; U2AF^{6}-AF^{26}, 100 nM) compared to U2AF^{65} alone (270 nM) was only about twofold. This suggests that

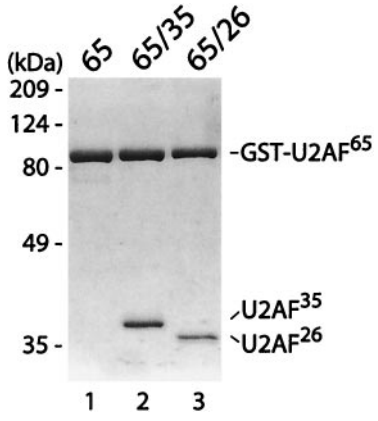

FIG. 4. Expression and purification of U2AF complexes. Approximately 2 μg of recombinant U2AF^{65} (lane 1), U2AF^{65}-U2AF^{35} (lane 2), or U2AF^{65}-U2AF^{26} (lane 3) purified from baculovirus-infected Sf9 cells was resolved on a sodium dodecyl sulfate–10% polyacrylamide gel and stained with Coomassie brilliant blue.

U2AF^{26}, similar to U2AF^{35}, functions to enhance the binding of U2AF^{65} to weak 3′ splice sites.

To determine whether the ability of U2AF^{26} to enhance U2AF^{65} binding involves an interaction between U2AF^{26} and the 3′ splice site AG, we generated two 100-nt RNA substrates. Both substrates contain a variant of the β-globin pre-mRNA in which the pyrimidine tract was weakened by introducing two purines. However, one substrate contains the 3′ splice site AG dinucleotide, and the other lacks this sequence. Both U2AF^{35} and U2AF^{26} enhanced the affinity of U2AF^{65} for the substrate containing the AG by 15- to 20-fold (U2AF^{65}, 750 nM; U2AF^{65}-U2AF^{35}, 50 nM; U2AF^{65}-U2AF^{26}, 35 nM) (Fig. 6C). However, when the AG is deleted, the effect of U2AF^{35} and U2AF^{26} on U2AF binding is reduced to only fourfold (U2AF^{65}, 60 nM; U2AF65-U2AF35, 15 nM; U2AF^{65}-U2AF^{26}, 16 nM) (Fig. 6D). Interestingly, each of the protein preparations has a higher affinity for the RNA lacking the AG than the RNA containing the AG (Fig. 6C and D). We believe that this may be due to differences in the RNA structure that present the pyrimidine tract of the RNA lacking the AG in a conformation more accessible to binding. However, the relative affinities of the U2AF heterodimers compared to U2AF^{65} alone are much higher for the RNA containing the AG (15- to 20-fold) than for the RNA lacking the AG (4-fold). These results show that U2AF^{26}, like U2AF^{35}, stabilizes the binding of U2AF^{65} to weak pyrimidine tracts. Given the similarity in the sequence of the RRM of U2AF^{35} and U2AF^{26}, the well-documented interaction between U2AF^{35} and the 3′ splice site AG (21, 40, 47), and the nearly identical behavior of the two proteins in our assays, we believe that U2AF^{26}, like U2AF^{35}, can directly interact with the AG dinucleotide at the 3′ splice site.

DISCUSSION

Here, we report the identification and characterization of a protein designated U2AF^{26}, which is highly related to the splicing factor U2AF^{35}. Although the function of U2AF^{35} had been elusive for many years, it has recently been shown to function by interacting with the 3′ splice site AG and thus stabilizing the binding of U2AF^{65} to weak pyrimidine tracts (21, 40, 47). Our results demonstrate that U2AF^{26} is a nuclear protein that interacts with U2AF^{65} and enhances its binding to weak pyrimidine tracts. We further show that this activity likely involves an interaction between U2AF^{26} and the AG dinucleotide at the 3′ splice site. Thus, U2AF^{26} functions as a splicing factor much in the same way as U2AF^{35}.

Orthologues of U2AF^{26} do not appear to exist in lower eukaryotes, *Drosophila*, *C. elegans*, plants, or vertebrates such as *Xenopus* or zebra fish (data not shown). By contrast, ESTs more similar to U2AF^{26} than U2AF^{35} are also found in rat (AW142126, AW142127, and AA848227), pig (BF193007 and BF440828), and cow (BE589680) (GenBank accession numbers are given in parentheses). In addition, humans contain a *U2AF^{26}* gene located on chromosome 19 (NT_011296), and several human ESTs in GenBank correspond to this gene. A comparison of the mouse and human *U2AF^{26}* gene revealed that the exon-intron boundaries are located in the same positions as in the human *U2AF^{35}* gene, although the introns are much smaller in the *U2AF^{26}* gene. In addition, the exon sequences of the human and mouse *U2AF^{26}* genes are 90%

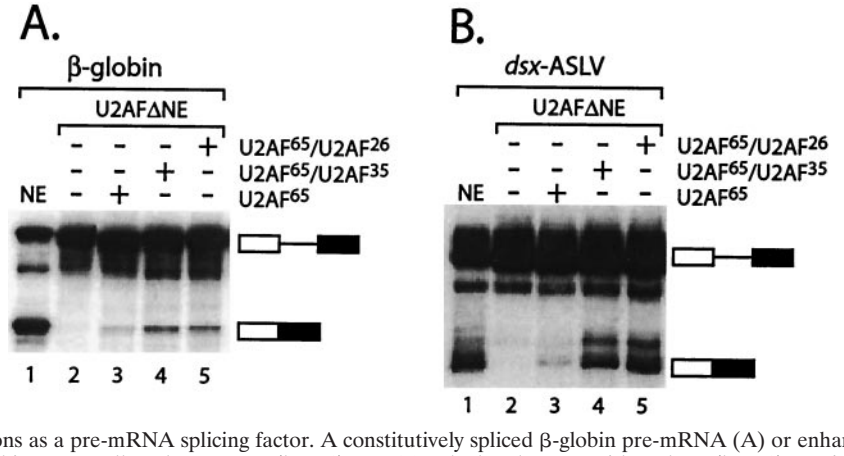

FIG. 5. U2AF^{26} functions as a pre-mRNA splicing factor. A constitutively spliced β-globin pre-mRNA (A) or enhancer-dependent *dsx*-ASLV pre-mRNA (B) was spliced in HeLa cell nuclear extract (lane 1) or U2AF-depleted extract either alone (lane 2) or with 25 nM U2AF^{65} (lane 3), U2AF^{65}-U2AF^{35} (lane 4), or U2AF^{65}-U2AF^{26} (lane 5).

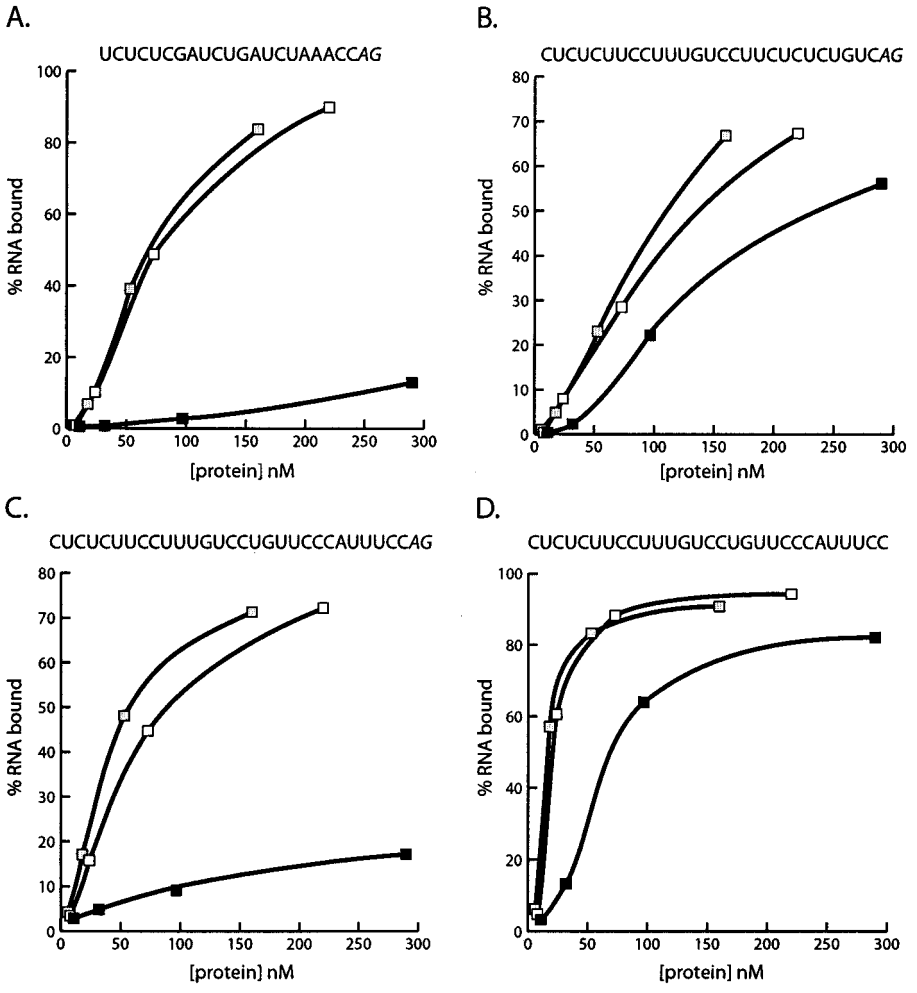

FIG. 6. U2AF^{26} enhances the binding of U2AF^{65} to weak 3′ splice sites. The binding of the various U2AF preparations was compared on RNAs containing the weak *dsx* 3′ splice site (A) or the strong β-globin 3′ splice site (B). In addition, U2AF binding was measured on RNAs containing a variant of the β-globin 3′ splice site that either contained (C) or lacked (D) the 3′ splice site AG dinucleotide. The binding reaction mixtures were resolved with nondenaturing polyacrylamide gels and quantitated with a Molecular Dynamics PhosphorImager. The protein concentrations used in the binding experiments were 10, 32, 97, and 290 nM for U2AF^{65} (black squares); 8, 24, 73, and 220 nM for U2AF^{65}-U2AF^{35} (white squares); and 6, 18, 53, and 160 nM for U2AF^{65}-U2AF^{26} (gray squares). All of the RNAs used in these experiments were approximately 100 nt. Only the sequence of the 3′ splice site of each RNA is shown above the graph.

identical at the nucleotide level; the majority of the differences are neutral, third position changes. Thus, the gene encoding U2AF^{26} appears to have recently arisen in mammals by duplication of the *U2AF^{35}* gene. Given the high degree of similarity between the human and mouse *U2AF^{26}* genes, and the fact that human and rodents diverged approximately 96 million years ago (24), it is likely that U2AF^{26} has a unique function.

Although proteins related to U2AF^{35} have been described previously, U2AF^{26} is the only one that has been shown to function in a manner similar to U2AF^{35}. U2AF1-RS1 and U2AF1-RS2/Urp are 94% identical to one another and contain stretches that are approximately 50% identical to portions of U2AF^{35} (33). Like U2AF^{26}, Urp interacts with U2AF^{65} in a manner likely to be mutually exclusive with U2AF^{35} (33). However, Urp contains an RS domain, interacts with SR proteins, and is functionally distinct from U2AF^{35} because U2AF^{35} cannot complement Urp-depleted extracts (33). In addition to U2AF1-RS1 and U2AF1-RS2–Urp, four proteins related to U2AF^{35} were recently identified in the draft of the human genome sequence (34). These observations, together with our data, suggest that mammalian cells contain multiple U2AF complexes.

In addition to its role in constitutive splicing, U2AF^{35} has been shown in some studies to be required for efficient enhancer-dependent splicing (11, 12, 46, 49). One model proposes that SR proteins bound to ESEs act to recruit U2AF to the upstream 3′ splice site (3, 11, 36, 46, 49). This activity is thought to require interactions between SR proteins and the RS domain of U2AF^{35} (40, 49). However, studies with *Drosophila* have shown that although the small U2AF subunit, dU2AF^{38}, and its interaction with the large U2AF subunit are essential for viability (29, 30), its RS domain is not (28). Moreover, the alternative splicing of the enhancer-dependent *dsx* pre-mRNA is not affected in flies lacking the dU2AF^{38} RS

domain (28). Additionally, both the *C. elegans* and *S. pombe* small U2AF subunits naturally lack RS domains (37, 48). Together these observations suggest that enhancer-dependent splicing does not require interactions between enhancer-bound SR proteins and the RS domain of the small U2AF subunit. Our results support this model because U2AF^{26}, which lacks an RS domain, can functionally substitute for U2AF^{35} in enhancer-dependent splicing. It remains to be determined whether U2AF^{26} can directly interact with SR proteins.

Although U2AF^{26} appears functionally identical to U2AF^{35} in our assays, it is our anticipation that U2AF^{26} will have a unique function in vivo that we have yet to uncover. The observation that U2AF^{35} and U2AF^{26} are differentially expressed raises the possibility that U2AF^{26} functions in tissue-specific alternative splicing. Interestingly, with respect to U2AF^{35}, U2AF^{26} is expressed most strongly in the brain where the occurrence of alternative splicing is unusually high (9). Thus, alternative splicing may be affected by the regulated expression of U2AF^{26}.

What could be the functional differences between these two proteins? One possibility is that the U2AF heterodimers containing either U2AF^{35} or U2AF^{26} could recognize distinct, yet overlapping sets of 3′ splice sites. In support of this model, it is interesting that in contrast to the RNP-1 sequences, the RNP-2 motif is slightly different between the two proteins. In particular, the first residue of RNP-2 is an Ala in U2AF^{35} and a Val in U2AF^{26}. Based on modeling with other RRM-RNA crystal structures, it was proposed that Ala47 of U2AF^{35} may directly contact RNA (17). Thus, although both U2AF^{26} and U2AF^{35} appear to bind RNA, and recognize the AG dinucleotide at the 3′ splice site, we believe that the binding specificities of the two heterodimers may be slightly different. Thus, it is possible that the two heterodimers recognize both common and distinct sets of 3′ splice sites. In this scenario, differences in the expression levels of U2AF^{35} and U2AF^{26} could influence alternative splicing because the two U2AF isoforms would recognize and activate different 3′ splice sites.

Alternatively, it is possible that the U2AF^{65}-U2AF^{26} and U2AF^{65}-U2AF^{35} heterodimers interact differently with splicing regulatory factors. The crystal structure of the core U2AF^{65}-U2AF^{35} heterodimer revealed that helix A in the U2AF^{35} RRM is unusually long and amphipathic (17). In particular, glutamate residues 73, 76, 80, 83, 84, 88, 90, and 91; Asp79; and Thr87 (which would be negatively charged if phosphorylated) of U2AF^{35} all reside on one side of helix A. Kielkopf et al. point out that the highly negatively charged face of this helix may be an interaction surface for other splicing factors—possibly the basic RS domains contained in SR proteins (17). Thus, changes in the residues contained in this alpha helix might alter the interactions between U2AF and other splicing factors. Interestingly, in U2AF^{26}, Glu80 is changed to Asn and Glu90 is changed to Gln. These uncharged amino acids both reside on the negatively charged surface of helix A and will change its electrostatic surface, possibly affecting the types of protein interactions in which U2AF^{26} can participate. Thus, U2AF may have distinct, yet overlapping, responses to different splicing factors depending on whether U2AF^{35} or U2AF^{26} is associated with U2AF^{65}. Given that the levels of U2AF^{26} and U2AF^{35} fluctuate with respect to one another in different tissues, we imagine that the relative concentrations of the different U2AF heterodimer isoforms play an important role in determining the splicing patterns of a number of pre-mRNAs.

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