

Characterization of U2AF²⁶, a Splicing Factor Related to U2AF³⁵

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Received 22 June 2001/Returned for modification 17 July 2001/Accepted 5 October 2001

The essential splicing factor U2AF (U2 auxiliary factor) is a heterodimer composed of 65-kDa (U2AF⁶⁵) and 35-kDa (U2AF³⁵) subunits. U2AF³⁵ has multiple functions in pre-mRNA splicing. First, U2AF³⁵ has been shown to function by directly interacting with the AG at the 3' splice site. Second, U2AF³⁵ is thought to play a role in the recruitment of U2AF⁶⁵ 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 U2AF³⁵ 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 U2AF³⁵, designated U2AF²⁶. The N-terminal 187 amino acids of U2AF³⁵ and U2AF²⁶ are nearly identical. However, the C-terminal domain of U2AF²⁶ lacks many characteristics of the U2AF³⁵ RS domain and, therefore, might be incapable of interacting with SR proteins. We show that U2AF²⁶ can associate with U2AF⁶⁵ and can functionally substitute for U2AF³⁵ 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 U2AF²⁶ functions by enhancing the binding of U2AF⁶⁵ to weak 3' splice sites. These studies identify U2AF²⁶ 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 U2AF³⁵, U2AF²⁶ 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 (U2AF⁶⁵) and 35-kDa (U2AF³⁵) subunits (42). U2AF⁶⁵ contains an N-terminal arginine-serine-rich (RS) domain and three RNA recognition motifs (RRM) (43). U2AF⁶⁵ is an essential splicing factor in vitro and is required for viability in

Drosophila melanogaster (15), *Caenorhabditis elegans* (20) and *Schizosaccharomyces pombe* (25). U2AF⁶⁵ 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 U2AF³⁵ in pre-mRNA splicing has been more controversial than that of U2AF⁶⁵. U2AF³⁵ 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 U2AF³⁵ in pre-mRNA splicing has been unclear because some studies have shown that U2AF³⁵ is dispensable for splicing in vitro (14, 41), while other studies indicate that U2AF³⁵ 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 U2AF⁶⁵ (29). One function of U2AF³⁵ in pre-mRNA splicing was recently clarified when three groups demonstrated that U2AF³⁵ associates with the AG at the 3' splice site (21, 40, 47).

In addition to constitutive splicing, U2AF³⁵ 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 U2AF⁶⁵ 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 U2AF³⁵ (39, 49). However, other studies indicate that ESEs may function through a mechanism that requires neither U2AF recruitment (14, 19) nor U2AF³⁵ (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 dU2AF³⁸ lacking its RS domain as the sole source of dU2AF³⁸ is not only viable but properly regulates the alternative splicing of the enhancer-dependent *doublesex* pre-mRNA (28). Thus, at least in non-vertebrate 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, *U2AF²⁶*, capable of encoding a protein product very similar to U2AF³⁵. The N-terminal portion of the U2AF²⁶ polypeptide, which contains two zinc fingers, a non-canonical RRM, and a U2AF⁶⁵ interaction domain, is 89% identical to U2AF³⁵. However, the C-terminal domain of U2AF²⁶ is quite different from the C-terminal domain of U2AF³⁵—most of the RS dipeptides, as well as the entire glycine tract present in U2AF³⁵, are absent in U2AF²⁶. We find that the relative levels of U2AF³⁵ and U2AF²⁶ mRNAs vary in different mouse tissues. U2AF²⁶ is a nuclear protein that localizes to speckles and can physically associate with U2AF⁶⁵. Biochemical experiments demonstrate that U2AF²⁶ functions as a pre-mRNA splicing factor and can functionally substitute for U2AF³⁵ in constitutive splicing *in vitro*. Interestingly, U2AF²⁶ can also replace U2AF³⁵ 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 U2AF²⁶ enhances the binding of U2AF⁶⁵ to weak 3' splice sites. These studies identify U2AF²⁶ as a mammalian splicing factor that may participate in the regulation of alternative splicing.

MATERIALS AND METHODS

Cloning of U2AF²⁶. We identified a cDNA fragment corresponding to the N terminus of U2AF²⁶ 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 U2AF²⁶ 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 U2AF³⁵ cDNA was cloned from total brain RNA by reverse transcription-PCR. Using the mouse U2AF²⁶ cDNA as a probe, we screened a mouse 129/SvEV Tac f BR genomic library (Stratagene) for the U2AF²⁶ gene. Using overlapping genomic clones, we were able to assemble a contig containing the complete U2AF²⁶ 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 ³²P-labeled DNA probes. The U2AF²⁶ transcripts were detected with a full-length cDNA probe, while the U2AF³⁵ 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 U2AF³⁵ and U2AF²⁶ 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 U2AF³⁵ and U2AF²⁶ 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 U2AF²⁶ to U2AF³⁵.

Indirect immunofluorescence. HeLa cells were transfected with 1 μg of pcDNA-mU2AF²⁶/V5, which encoded murine U2AF²⁶ (mU2AF²⁶) containing a C-terminal V5 epitope tag, using Fugene6 (Boehringer Mannheim). In addition,

cells were transfected with control plasmids expressing either human U2AF³⁵ (hU2AF³⁵) 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. His₆-mU2AF²⁶ and His₆-hU2AF³⁵ were cloned into the baculovirus expression vector, pFastBac-1 (Gibco). The N-terminal His₆ tag was added by PCR. To construct a virus encoding GST-hU2AF⁶⁵, hU2AF⁶⁵ was first cloned into pGEXT-4T (Amersham-Pharmacia), amplified by PCR to isolate the fragment encoding GST-U2AF⁶⁵, and subsequently cloned into pFastBac-1. Baculoviruses encoding these proteins were produced as described by the manufacturer (Gibco). The U2AF⁶⁵-U2AF²⁶ and U2AF⁶⁵-U2AF³⁵ heterodimers were produced by coinfecting the Sf9 cells with the respective viruses, whereas U2AF⁶⁵ 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. U2AF⁶⁵ 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. ³²P-labeled RNAs were synthesized with either SP6 or T7 RNA polymerase.

In vitro-splicing assays. HeLa cell nuclear extract was depleted of U2AF⁶⁵ and U2AF³⁵ 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 U2AF⁶⁵ and U2AF³⁵ 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 ³²P-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 U2AF⁶⁵ 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 *K₁* and *K₂* are the estimated equilibrium association constants for U2AF⁶⁵ and U2AF³⁵ or U2AF²⁶, *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 U2AF²⁶ and its similarity to U2AF³⁵. A cDNA fragment encoding a protein similar to U2AF³⁵ 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

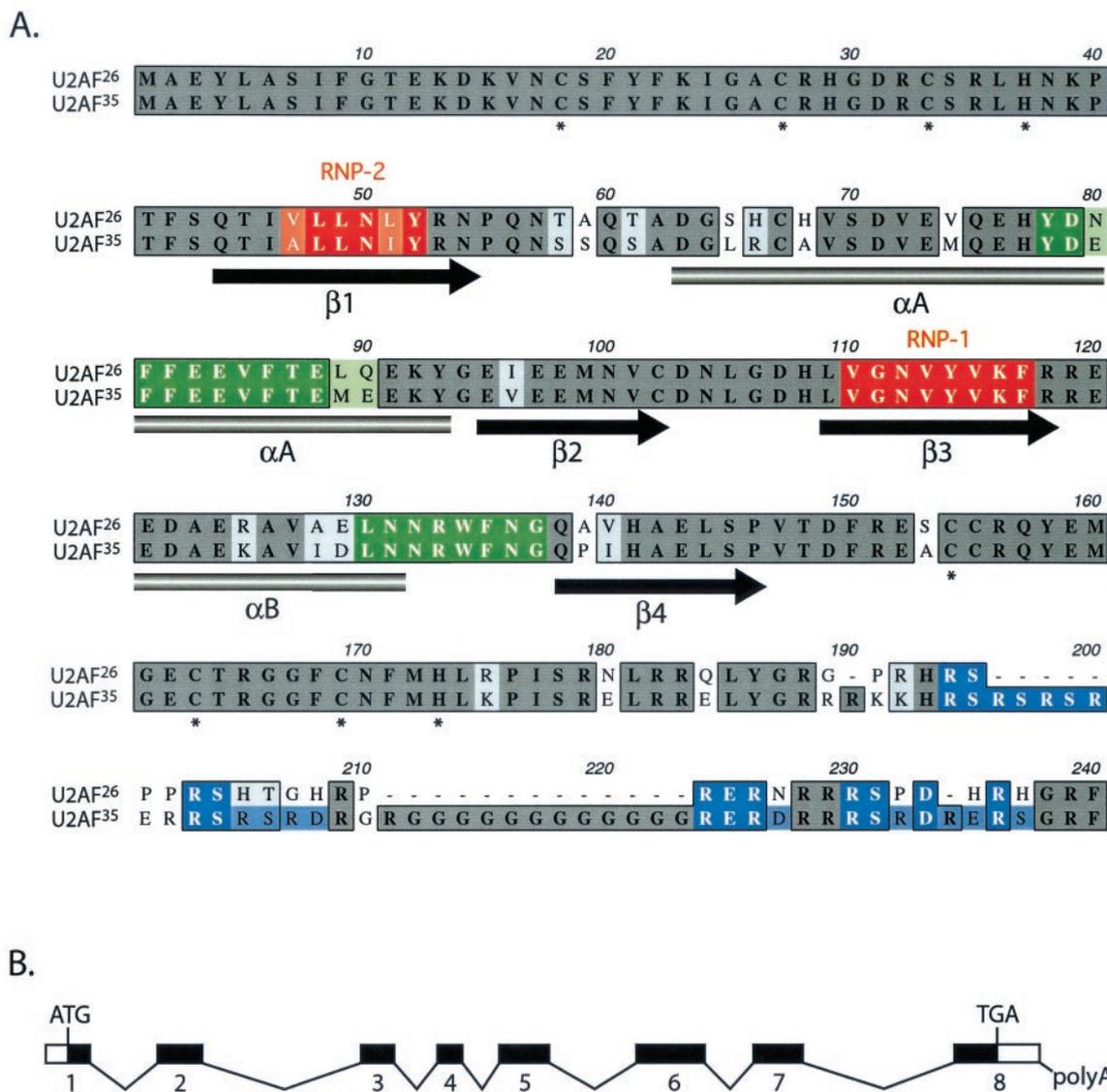


FIG. 1. Sequence comparison of mU2AF²⁶ and U2AF³⁵ proteins and organization of the *U2AF²⁶* gene. (A) U2AF³⁵ and U2AF²⁶ share 89% sequence identity within the first 187 residues of the proteins. The dark grey boxes indicate identical amino acids, and the light grey boxes indicate conserved amino acids. 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 U2AF⁶⁵ are highlighted in green. In addition, RS, RE, or RD dipeptides in the C terminus are highlighted in blue. (B) Organization of the mouse *U2AF²⁶* gene.

weight of 26 kDa that is 76% identical to mU2AF³⁵. We therefore designate this protein U2AF²⁶.

U2AF³⁵ and U2AF²⁶ 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 U2AF³⁵ and U2AF²⁶ (Fig. 1A). However, the RNP-2 motif of U2AF²⁶ contains two amino acid substitutions with respect to U2AF³⁵—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 U2AF²⁶, like U2AF³⁵, has the ability to bind RNA (21, 40, 47). In addition, the majority of the residues in U2AF³⁵

that directly interact with U2AF⁶⁵ (17, 44) are conserved in U2AF²⁶. For example, the crystal structure of the U2AF³⁵-U2AF⁶⁵ heterodimer (17) revealed that Glu80, Phe81, Glu84, Glu88, Arg133, and Phe135 of U2AF³⁵ are in intimate contact with U2AF⁶⁵. Of these residues, only Glu80 is different in U2AF²⁶ (changed to an Asn). Most importantly, Trp134, perhaps the most critical residue involved in the U2AF⁶⁵-U2AF³⁵ interaction (17), is conserved in U2AF²⁶. This suggests that U2AF²⁶ most likely can physically interact with U2AF⁶⁵.

In contrast to the highly conserved N terminus, the C terminus of U2AF²⁶ differs markedly from that of U2AF³⁵. The U2AF³⁵ C-terminal domain contains an RS domain and a glycine tract (44). U2AF³⁵ orthologues have been cloned from *Drosophila* (30), *C. elegans* (48), and *S. pombe* (37). The glycine

tract present in U2AF³⁵ is conserved in both the *Drosophila* and *C. elegans* U2AF³⁵ orthologues, dU2AF³⁸ and CeU2AF³⁵, but not the *S. pombe* orthologue, SpU2AF²³. In addition, neither CeU2AF³⁵ nor SpU2AF²³ contain an RS domain, whereas dU2AF³⁸ does. The U2AF³⁵ 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 U2AF²⁶ 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 U2AF²⁶ are adjacent to one another. The RS domain of U2AF³⁵ 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 U2AF²⁶ suggests that both of these activities are altered in U2AF²⁶.

Cloning of the U2AF²⁶ gene. To compare the genomic organization of the U2AF²⁶ gene with that of U2AF³⁵, we cloned and sequenced the gene encoding U2AF²⁶ from a mouse genomic DNA library. The U2AF²⁶ 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 U2AF²⁶ gene are identical to those in the human U2AF³⁵ gene located on chromosome 21 (18).

U2AF²⁶ is differentially expressed. To examine the expression of U2AF²⁶ in comparison to U2AF³⁵, 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 U2AF²⁶ to U2AF³⁵ 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 U2AF²⁶ and U2AF³⁵ mRNAs varies in different tissues (Fig. 2B). For example, the ratio of U2AF²⁶ to U2AF³⁵ is highest in brain (~3) and lowest in liver (~0.5). Thus, U2AF²⁶ is differentially expressed in various mouse tissues.

Subcellular localization of U2AF²⁶. To determine the subcellular localization of U2AF²⁶, HeLa cells were transfected with an expression vector encoding U2AF²⁶ containing a C-terminal V5 epitope tag. As a control, HeLa cells were transfected with expression vectors encoding U2AF³⁵ or the SR protein SF2/ASF, each containing a C-terminal V5 epitope tag. Indirect immunofluorescence revealed that, as expected, U2AF³⁵ (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, U2AF²⁶ was observed to be localized exclusively to the nucleus and associated with nuclear speckles (Fig. 3). These results demonstrate that U2AF²⁶, like U2AF³⁵, is a nuclear protein concentrated in speckles and thus is properly localized to function as a pre-mRNA splicing factor.

Expression and purification of U2AF⁶⁵-U2AF²⁶. To examine whether U2AF²⁶ might function as a pre-mRNA splicing factor, we first needed to express and purify the protein. Attempts to purify recombinant U2AF²⁶ alone from *Escherichia coli* or Sf9 cells yielded only insoluble protein. We therefore coexpressed His-tagged U2AF²⁶ and glutathione *S*-transferase (GST)-tagged U2AF⁶⁵ in Sf9 cells using baculovirus expression vectors. In parallel, the conventional GST-U2AF⁶⁵-His-U2AF³⁵ heterodimer, as well as GST-U2AF⁶⁵ 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 U2AF²⁶ or U2AF³⁵. Secondly, the eluate from the Ni-NTA column was passed over a GST-agarose column to purify the GST-U2AF⁶⁵. The eluate from the GST-agarose column was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. As expected, U2AF⁶⁵ and U2AF³⁵ copurified by this method in a 1:1 stoichiometry (Fig. 4, lane 2). We found that U2AF⁶⁵ and U2AF²⁶ 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 U2AF²⁶ and U2AF⁶⁵ are present in a complex (data not shown). These results demonstrate that U2AF²⁶ can physically interact with U2AF⁶⁵, suggesting that U2AF²⁶ has the ability to modify the activity of U2AF⁶⁵ in pre-mRNA splicing.

U2AF²⁶ is a pre-mRNA splicing factor that can functionally replace U2AF³⁵ in vitro. Given the similarities between U2AF²⁶ and U2AF³⁵, we were interested in testing whether U2AF²⁶ could function as a pre-mRNA splicing factor and, if so, comparing its activity to that of U2AF³⁵. We therefore compared the ability of U2AF⁶⁵, U2AF⁶⁵-U2AF²⁶, or U2AF⁶⁵-U2AF³⁵ to restore splicing to HeLa cell nuclear extracts depleted of endogenous U2AF⁶⁵ and U2AF³⁵ (Δ NE). Previous work had shown that U2AF³⁵ enhances the ability of U2AF⁶⁵ to restore splicing in Δ NE (11, 12, 49). We first tested whether U2AF²⁶ could function in constitutive splicing. As shown in Fig. 5A, U2AF⁶⁵ alone weakly restores the splicing of β -globin pre-mRNA in Δ NE (Fig. 5A, lane 3). However, both U2AF⁶⁵-U2AF³⁵ and U2AF⁶⁵-U2AF²⁶ were approximately twofold more effective in restoring splicing of this substrate than U2AF⁶⁵ alone (lanes 4 and 5). Similar results were observed for the adenovirus major late pre-mRNA (data not shown). These observations indicate that U2AF²⁶ can function as a pre-mRNA splicing factor and that it can functionally substitute for U2AF³⁵ in constitutive splicing.

We next tested whether U2AF²⁶ 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). U2AF⁶⁵ alone weakly restored splicing of this pre-mRNA in Δ NE (Fig. 5B, lane 3). By contrast, both U2AF⁶⁵-U2AF³⁵ and U2AF⁶⁵-U2AF²⁶ were five- to sixfold more effective in restoring splicing of the *dsx*-ASLV pre-mRNA in Δ NE than U2AF⁶⁵ alone (lanes 4 and 5). Thus, U2AF²⁶ can functionally substitute for U2AF³⁵ in both constitutive splicing and enhancer-dependent splicing. These

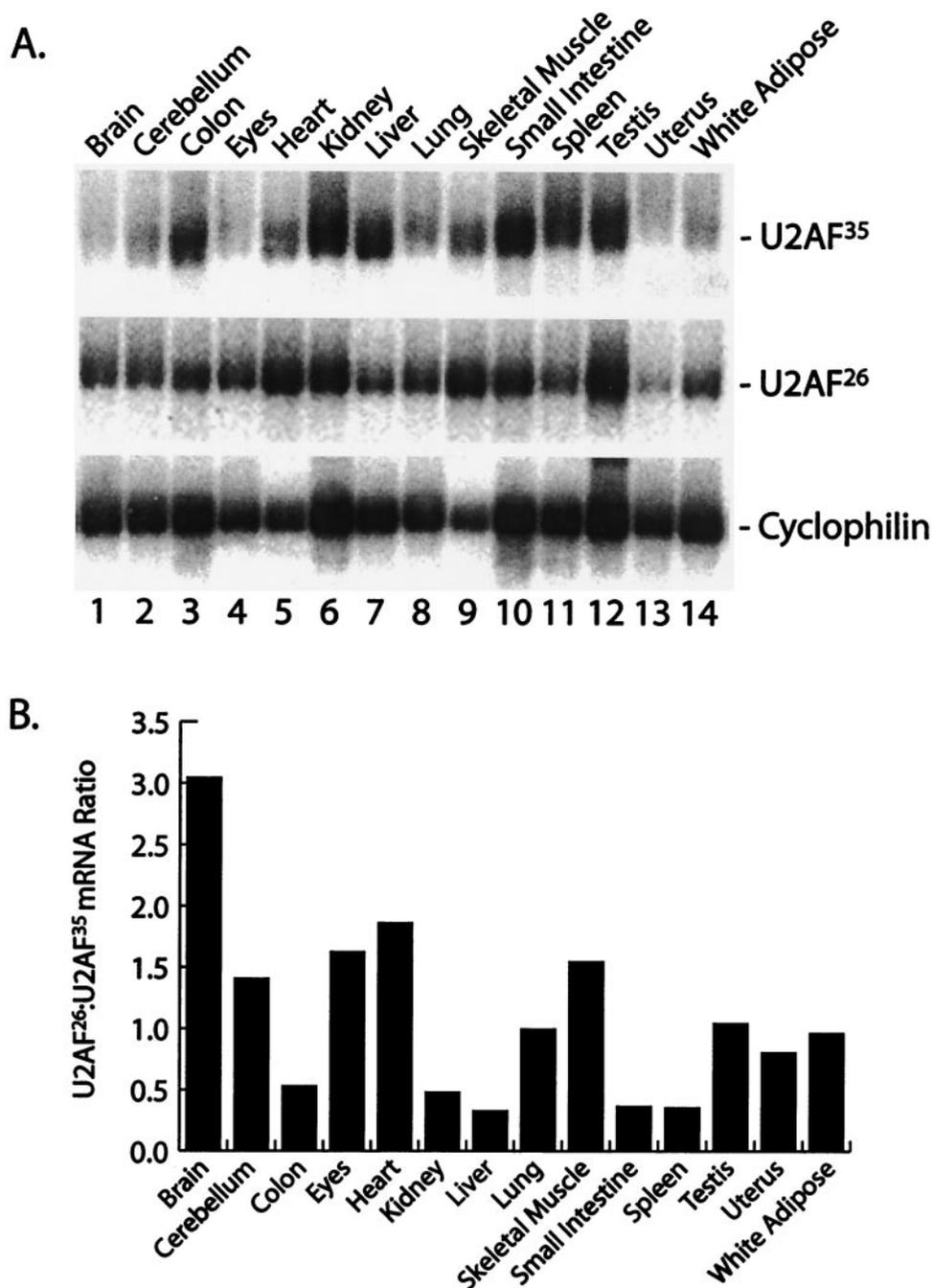


FIG. 2. Comparison of the expression patterns of U2AF²⁶ and U2AF³⁵ in mouse tissues. Northern blots containing poly(A)⁺ RNA isolated from the indicated mouse tissues was hybridized with probes specific to the C-terminal domain of mU2AF³⁵ (top), full-length mU2AF²⁶ (middle), or a cyclophilin control (bottom). (B) The plot depicts the ratio of U2AF²⁶ to U2AF³⁵ 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.

U2AF²⁶ enhances the binding of U2AF⁶⁵ to weak 3' splice sites. Our data demonstrate that U2AF²⁶ can functionally substitute for U2AF³⁵ in both constitutive and enhancer-dependent splicing. We next wanted to determine the basis for the activity of U2AF²⁶. Given that U2AF²⁶ appeared to be functionally similar to U2AF³⁵, 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 U2AF³⁵ (21, 40, 47), we thought U2AF²⁶ might act by stabilizing the binding of U2AF⁶⁵ on weak 3' splice sites and that this activity might involve an interaction between U2AF²⁶ 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,

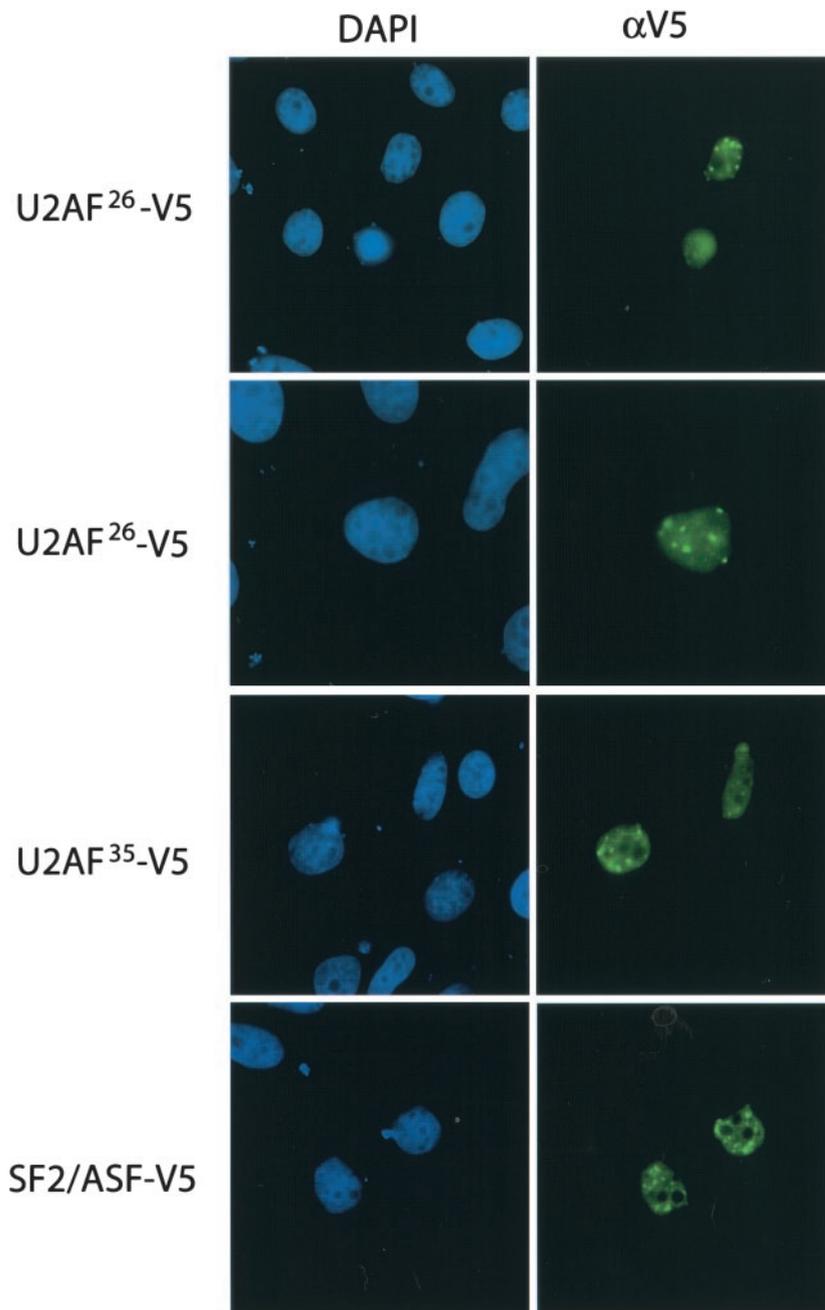


FIG. 3. Immunolocalization of U2AF²⁶. HeLa cells were transfected with expression vectors encoding C-terminal V5-epitope tagged U2AF²⁶ (top two rows), U2AF³⁵ (third row), or SF2/ASF (bottom row). The cells were fixed and probed with an anti-V5 primary antibody and a fluorescein isothiocyanate-tagged secondary antibody (right). In addition, the cells were stained with DAPI to visualize the nucleus (left). Two separate fields of U2AF²⁶ are shown (first and second rows). Speckles cannot be observed in the lower U2AF²⁶-positive cell in the top row because it is out of the plane of focus.

the U2AF⁶⁵-U2AF³⁵ heterodimer bound much more efficiently to a 100-nt RNA containing the weak *dsx* 3' splice site ($K_d = 65$ nM) than did U2AF⁶⁵ alone ($K_d = 2,000$ nM) (Fig. 6A). Surprisingly, the affinity of the U2AF⁶⁵-U2AF²⁶ heterodimer for the *dsx* 3' splice site ($K_d = 60$ nM) was nearly identical to that of the U2AF⁶⁵-U2AF³⁵ heterodimer (Fig. 6A). Thus, both U2AF³⁵ and U2AF²⁶ enhance the affinity of U2AF for the *dsx* 3' splice site by greater than 30-fold. How-

ever, the impact of U2AF³⁵ or U2AF²⁶ 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 U2AF⁶⁵ binding site (data not shown). In the case of the β -globin pyrimidine tract, the difference in affinity of the U2AF heterodimers (U2AF⁶⁵-U2AF³⁵, 120 nM; U2AF⁶⁵-U2AF²⁶, 100 nM) compared to U2AF⁶⁵ alone (270 nM) was only about twofold. This suggests that

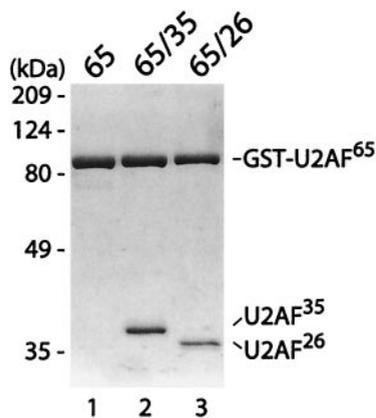


FIG. 4. Expression and purification of U2AF complexes. Approximately 2 μ g of recombinant U2AF⁶⁵ (lane 1), U2AF⁶⁵-U2AF³⁵ (lane 2), or U2AF⁶⁵-U2AF²⁶ (lane 3) purified from baculovirus-infected Sf9 cells was resolved on a sodium dodecyl sulfate–10% polyacrylamide gel and stained with Coomassie brilliant blue.

U2AF²⁶, similar to U2AF³⁵, functions to enhance the binding of U2AF⁶⁵ to weak 3' splice sites.

To determine whether the ability of U2AF²⁶ to enhance U2AF⁶⁵ binding involves an interaction between U2AF²⁶ 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 U2AF³⁵ and U2AF²⁶ enhanced the affinity of U2AF⁶⁵ for the substrate containing the AG by 15- to 20-fold (U2AF⁶⁵, 750 nM; U2AF⁶⁵-U2AF³⁵, 50 nM; U2AF⁶⁵-U2AF²⁶, 35 nM) (Fig. 6C). However, when the AG is deleted, the effect of U2AF³⁵ and U2AF²⁶ on U2AF binding is reduced to only fourfold (U2AF⁶⁵, 60 nM; U2AF⁶⁵-U2AF³⁵, 15 nM; U2AF⁶⁵-U2AF²⁶, 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 confor-

mation more accessible to binding. However, the relative affinities of the U2AF heterodimers compared to U2AF⁶⁵ 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 U2AF²⁶, like U2AF³⁵, stabilizes the binding of U2AF⁶⁵ to weak pyrimidine tracts. Given the similarity in the sequence of the RRM of U2AF³⁵ and U2AF²⁶, the well-documented interaction between U2AF³⁵ and the 3' splice site AG (21, 40, 47), and the nearly identical behavior of the two proteins in our assays, we believe that U2AF²⁶, like U2AF³⁵, can directly interact with the AG dinucleotide at the 3' splice site.

DISCUSSION

Here, we report the identification and characterization of a protein designated U2AF²⁶, which is highly related to the splicing factor U2AF³⁵. Although the function of U2AF³⁵ 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 U2AF⁶⁵ to weak pyrimidine tracts (21, 40, 47). Our results demonstrate that U2AF²⁶ is a nuclear protein that interacts with U2AF⁶⁵ and enhances its binding to weak pyrimidine tracts. We further show that this activity likely involves an interaction between U2AF²⁶ and the AG dinucleotide at the 3' splice site. Thus, U2AF²⁶ functions as a splicing factor much in the same way as U2AF³⁵.

Orthologues of U2AF²⁶ 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 U2AF²⁶ than U2AF³⁵ 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 U2AF²⁶ gene located on chromosome 19 (NT_011296), and several human ESTs in GenBank correspond to this gene. A comparison of the mouse and human U2AF²⁶ gene revealed that the exon-intron boundaries are located in the same positions as in the human U2AF³⁵ gene, although the introns are much smaller in the U2AF²⁶ gene. In addition, the exon sequences of the human and mouse U2AF²⁶ genes are 90%

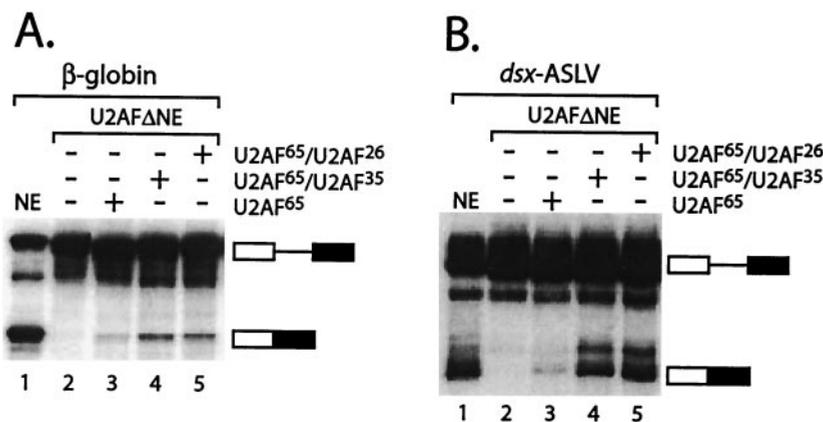


FIG. 5. U2AF²⁶ functions as a pre-mRNA splicing factor. A constitutively spliced β -globin pre-mRNA (A) or enhancer-dependent *dsx*-ASLV pre-mRNA (B) was spliced in HeLa cell nuclear extract (lane 1) or U2AF-depleted extract either alone (lane 2) or with 25 nM U2AF⁶⁵ (lane 3), U2AF⁶⁵-U2AF³⁵ (lane 4), or U2AF⁶⁵-U2AF²⁶ (lane 5).

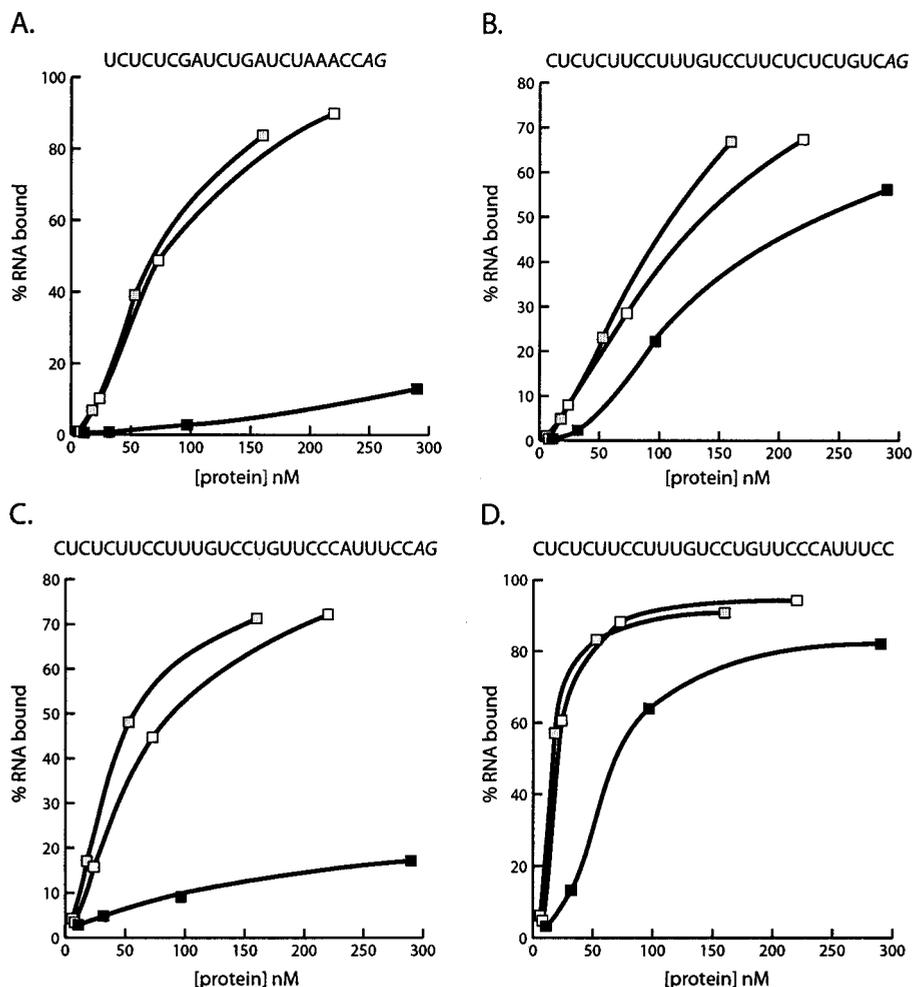


FIG. 6. U2AF²⁶ enhances the binding of U2AF⁶⁵ to weak 3' splice sites. The binding of the various U2AF preparations was compared on RNAs containing the weak *dsx* 3' splice site (A) or the strong β -globin 3' splice site (B). In addition, U2AF binding was measured on RNAs containing a variant of the 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 U2AF⁶⁵ (black squares); 8, 24, 73, and 220 nM for U2AF⁶⁵-U2AF³⁵ (white squares); and 6, 18, 53, and 160 nM for U2AF⁶⁵-U2AF²⁶ (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 U2AF²⁶ appears to have recently arisen in mammals by duplication of the U2AF³⁵ gene. Given the high degree of similarity between the human and mouse U2AF²⁶ genes, and the fact that human and rodents diverged approximately 96 million years ago (24), it is likely that U2AF²⁶ has a unique function.

Although proteins related to U2AF³⁵ have been described previously, U2AF²⁶ is the only one that has been shown to function in a manner similar to U2AF³⁵. U2AF1-RS1 and U2AF1-RS2/Urp are 94% identical to one another and contain stretches that are approximately 50% identical to portions of U2AF³⁵ (33). Like U2AF²⁶, Urp interacts with U2AF⁶⁵ in a manner likely to be mutually exclusive with U2AF³⁵ (33). However, Urp contains an RS domain, interacts with SR proteins, and is functionally distinct from U2AF³⁵ because U2AF³⁵ cannot complement Urp-depleted extracts (33). In addition to

U2AF1-RS1 and U2AF1-RS2-Urp, four proteins related to U2AF³⁵ 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, U2AF³⁵ 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 U2AF³⁵ (40, 49). However, studies with *Drosophila* have shown that although the small U2AF subunit, dU2AF³⁸, 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 dU2AF³⁸ 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 U2AF²⁶, which lacks an RS domain, can functionally substitute for U2AF³⁵ in enhancer-dependent splicing. It remains to be determined whether U2AF²⁶ can directly interact with SR proteins.

Although U2AF²⁶ appears functionally identical to U2AF³⁵ in our assays, it is our anticipation that U2AF²⁶ will have a unique function in vivo that we have yet to uncover. The observation that U2AF³⁵ and U2AF²⁶ are differentially expressed raises the possibility that U2AF²⁶ functions in tissue-specific alternative splicing. Interestingly, with respect to U2AF³⁵, U2AF²⁶ 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 U2AF²⁶.

What could be the functional differences between these two proteins? One possibility is that the U2AF heterodimers containing either U2AF³⁵ or U2AF²⁶ 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 U2AF³⁵ and a Val in U2AF²⁶. Based on modeling with other RRM-RNA crystal structures, it was proposed that Ala47 of U2AF³⁵ may directly contact RNA (17). Thus, although both U2AF²⁶ and U2AF³⁵ 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 U2AF³⁵ and U2AF²⁶ could influence alternative splicing because the two U2AF isoforms would recognize and activate different 3' splice sites.

Alternatively, it is possible that the U2AF⁶⁵-U2AF²⁶ and U2AF⁶⁵-U2AF³⁵ heterodimers interact differently with splicing regulatory factors. The crystal structure of the core U2AF⁶⁵-U2AF³⁵ heterodimer revealed that helix A in the U2AF³⁵ 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 U2AF³⁵ 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 U2AF²⁶, 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 U2AF²⁶ can participate. Thus, U2AF may have distinct, yet overlapping, responses to different splicing factors depending on whether U2AF³⁵ or U2AF²⁶ is associated with U2AF⁶⁵. Given that the levels of U2AF²⁶ and U2AF³⁵ 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.

ACKNOWLEDGMENTS

This work was primarily conducted in the laboratory of Steve McKnight in the Department of Biochemistry at UT Southwestern Medical Center. We thank Steve McKnight for extensive advice, encouragement, and support. We also thank J. Repa for assistance with Northern blots, J. Rutter for assistance with protein purification, K. Hertel for assistance with K_d calculations, R. Reed for the β -globin plasmids, and K. Lynch for comments on the manuscript.

This work was primarily funded by NIH grant 5 R37 MH59388-03 and an unrestricted endowment fund given to Steve McKnight by an anonymous donor. This work was also supported by NIH grant R01 GM62516-01 (to B.R.G.).

REFERENCES

- Birney, E., S. Kumar, and A. R. Krainer. 1993. Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.* **21**:5803–5816.
- Blencowe, B. J. 2000. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem. Sci.* **25**:106–110.
- Bouck, J., X.-D. Fu, A. M. Skalka, and R. A. Katz. 1998. Role of the constitutive splicing factors U2AF⁶⁵ and SAP49 in suboptimal RNA splicing of novel retroviral mutants. *J. Biol. Chem.* **273**:15169–15176.
- Burge, C. B., T. Tuschl, and P. A. Sharp. 1999. Splicing of precursors to mRNAs by the spliceosomes, p. 525–560. *In* R. F. Gesteland, T. R. Cech, and J. F. Atkins (ed.), *The RNA world*, 2nd. ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
- Caceres, J. F., T. Misteli, G. R. Screaton, D. L. Spector, and A. R. Krainer. 1997. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *J. Cell Biol.* **138**:225–238.
- Das, R., Z. Zhou, and R. Reed. 2000. Functional association of U2 snRNP with the ATP-independent spliceosomal complex E. *Mol. Cell* **5**:779–787.
- Gama-Carvalho, M., M. P. Carvalho, A. Kehlenbach, J. Valcárcel, and M. Carmo-Fonseca. 2001. Nucleocytoplasmic shuttling of heterodimeric splicing factor U2AF. *J. Biol. Chem.* **276**:13104–13112.
- Gozani, O., J. Potashkin, and R. Reed. 1998. A potential role for U2AF-SAP 155 interactions in recruiting U2 snRNP to the branch site. *Mol. Cell. Biol.* **18**:4752–4760.
- Grabowski, P. J., and D. L. Black. 2001. Alternative RNA splicing in the nervous system. *Prog. Neurobiol.* **65**:289–308.
- Graveley, B. R. 2000. Sorting out the complexity of SR protein functions. *RNA* **6**:1197–1211.
- Graveley, B. R., K. J. Hertel, and T. Maniatis. 2001. The role of U2AF³⁵ and U2AF⁶⁵ in enhancer-dependent splicing. *RNA* **7**:806–818.
- Guth, S., C. Martínez, R. K. Gaur, and J. Valcárcel. 1999. Evidence for substrate-specific requirement of the splicing factor U2AF³⁵ and for its function after polypyrimidine tract recognition by U2AF⁶⁵. *Mol. Cell. Biol.* **19**:8263–8271.
- Hertel, K. J., and T. Maniatis. 1998. The function of multisite splicing enhancers. *Mol. Cell* **1**:449–455.
- Kan, J. L. C., and M. R. Green. 1999. Pre-mRNA splicing of IgM exons M1 and M2 is directed by a juxtaposed splicing enhancer and inhibitor. *Genes Dev.* **13**:462–471.
- Kanaar, R., S. E. Roche, E. L. Beall, M. R. Green, and D. C. Rio. 1993. The conserved pre-mRNA splicing factor U2AF from *Drosophila*: requirement for viability. *Science* **262**:569–573.
- Katz, R. A., and A. M. Skalka. 1990. Control of retroviral RNA splicing through maintenance of suboptimal processing signals. *Mol. Cell. Biol.* **10**:696–704.
- Kielkopf, C. L., N. A. Rodionova, M. R. Green, and S. K. Burley. 2001. A novel peptide recognition mode revealed by the X-ray structure of a core U2AF³⁵/U2AF⁶⁵ heterodimer. *Cell* **106**:595–605.
- Lalioti, M. D., A. Gos, M. R. Green, C. Rossier, M. A. Morris, and S. E. Antonarakis. 1996. The gene for human U2 snRNP auxiliary factor small 35-kDa subunit (U2AF1) maps to the progressive myoclonus epilepsy (EPM1) critical region on chromosome 21q22.3. *Genomics* **33**:298–300.
- Li, Y., and B. J. Blencowe. 1999. Distinct factor requirements for exonic splicing enhancer function and binding of U2AF to the polypyrimidine tract. *J. Biol. Chem.* **274**:35074–35079.
- MacMorris, M. A., D. A. Zorio, and T. Blumenthal. 1999. An exon that prevents transport of a mature mRNA. *Proc. Natl. Acad. Sci. USA* **96**:3813–3818.
- Merendino, L., S. Guth, D. Bilbao, C. Martínez, and J. Valcárcel. 1999. Inhibition of msl-2 splicing by Sex-lethal reveals interaction between U2AF³⁵ and the 3' splice site AG. *Nature* **402**:838–841.

22. **Misteli, T., and D. L. Spector.** 1998. The cellular organization of gene expression. *Curr. Opin. Cell Biol.* **10**:323–331.
23. **Moore, M. J.** 2000. Intron recognition comes of AGE. *Nat. Struct. Biol.* **7**:14–16.
24. **Nei, M., P. Xu, and G. Glazko.** 2001. Estimation of divergence times from multiprotein sequences for a few mammalian species and several distantly related organisms. *Proc. Natl. Acad. Sci. USA* **98**:2497–2502.
25. **Potashkin, J., K. Naik, and K. Wentz-Hunter.** 1993. U2AF homolog required for splicing in vivo. *Science* **262**:573–575.
26. **Reed, R.** 2000. Mechanisms of fidelity in pre-mRNA splicing. *Curr. Opin. Cell Biol.* **12**:340–345.
27. **Reed, R.** 1989. The organization of 3' splice-site sequences in mammalian introns. *Genes Dev.* **3**:2113–2123.
28. **Rudner, D. Z., K. S. Breger, and D. C. Rio.** 1998. Molecular genetic analysis of the heterodimeric splicing factor U2AF: the RS domain on either the large or small *Drosophila* subunit is dispensable in vivo. *Genes Dev.* **12**:1010–1021.
29. **Rudner, D. Z., R. Kanaar, K. S. Breger, and D. C. Rio.** 1998. Interaction between subunits of heterodimeric splicing factor U2AF is essential in vivo. *Mol. Cell. Biol.* **18**:1765–1773.
30. **Rudner, D. Z., R. Kanaar, K. S. Breger, and D. C. Rio.** 1996. Mutations in the small subunit of the *Drosophila* U2AF splicing factor cause lethality and developmental defects. *Proc. Natl. Acad. Sci. USA* **93**:10333–10337.
31. **Smith, C. W., and J. Valcárcel.** 2000. Alternative pre-mRNA splicing: the logic of combinatorial control. *Trends Biochem. Sci.* **25**:381–388.
32. **Staknis, D., and R. Reed.** 1994. SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. *Mol. Cell. Biol.* **14**:7670–7682.
33. **Tronchere, H., J. Wang, and X. D. Fu.** 1997. A protein related to splicing factor U2AF³⁵ that interacts with U2AF⁶⁵ and SR proteins in splicing of pre-mRNA. *Nature* **388**:397–400.
34. **Tupler, R., G. Perini, and M. R. Green.** 2001. Expressing the human genome. *Nature* **409**:832–833.
35. **Valcárcel, J., R. K. Gaur, R. Singh, and M. R. Green.** 1996. Interaction of U2AF⁶⁵ RS region with pre-mRNA of branch point and promotion base pairing with U2 snRNA. *Science* **273**:1706–1709.
36. **Wang, Z., H. M. Hoffmann, and P. J. Grabowski.** 1995. Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. *RNA* **1**:21–35.
37. **Wentz-Hunter, K., and J. Potashkin.** 1996. The small subunit of the splicing factor U2AF is conserved in fission yeast. *Nucleic Acids Res.* **24**:1849–1854.
38. **Witherell, G. W., H. N. Wu, and O. C. Uhlenbeck.** 1990. Cooperative binding of R17 coat protein to RNA. *Biochemistry* **29**:11051–11057.
39. **Wu, J. Y., and T. Maniatis.** 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell* **75**:1061–1070.
40. **Wu, S., C. M. Romfo, T. W. Nilsen, and M. R. Green.** 1999. Functional recognition of the 3' splice site AG by the splicing factor U2AF³⁵. *Nature* **402**:832–835.
41. **Zamore, P. D., and M. R. Green.** 1991. Biochemical characterization of U2 snRNP auxiliary factor: an essential pre-mRNA splicing factor with a novel intranuclear distribution. *EMBO J.* **10**:207–214.
42. **Zamore, P. D., and M. R. Green.** 1989. Identification, purification, and biochemical characterization of U2 small nuclear ribonucleoprotein auxiliary factor. *Proc. Natl. Acad. Sci. USA* **86**:9243–9247.
43. **Zamore, P. D., J. G. Patton, and M. R. Green.** 1992. Cloning and domain structure of the mammalian splicing factor U2AF. *Nature* **355**:609–614.
44. **Zhang, M., P. D. Zamore, M. Carmo-Fonseca, A. I. Lamond, and M. R. Green.** 1992. Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit. *Proc. Natl. Acad. Sci. USA* **89**:8769–8773.
45. **Zhou, Y. D., M. Barnard, H. Tian, X. Li, H. Z. Ring, U. Francke, J. Shelton, J. Richardson, D. W. Russell, and S. L. McKnight.** 1997. Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. *Proc. Natl. Acad. Sci. USA* **94**:713–718.
46. **Zhu, J., and A. R. Krainer.** 2000. Pre-mRNA splicing in the absence of an SR protein RS domain. *Genes Dev.* **14**:3166–3178.
47. **Zorio, D. A., and T. Blumenthal.** 1999. Both subunits of U2AF recognize the 3' splice site in *Caenorhabditis elegans*. *Nature* **402**:835–838.
48. **Zorio, D. A., and T. Blumenthal.** 1999. U2AF³⁵ is encoded by an essential gene clustered in an operon with RRM/cyclophilin in *Caenorhabditis elegans*. *RNA* **5**:487–494.
49. **Zuo, P., and T. Maniatis.** 1996. The splicing factor U2AF³⁵ mediates critical protein-protein interactions in constitutive and enhancer-dependent splicing. *Genes Dev.* **10**:1356–1368.