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Cloning and intracellular localization of the U2 small nuclear ribonucleoprotein auxiliary factor small subunit

(splicing/spliceosome/RNA binding/arginine, serine rich/coiled body)

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ABSTRACT **U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), an essential mammalian splicing factor, is composed of two subunits: a 65-kDa protein ($U2AF^{65}$), which binds the pre-mRNA polypyrimidine tract and is required for *in vitro* splicing, and an associated 35-kDa protein ($U2AF^{35}$). Here we report the isolation of a cDNA encoding $U2AF^{35}$. $U2AF^{35}$ contains sequence motifs found in several mammalian pre-mRNA splicing factors. We show directly that $U2AF^{65}$ and $U2AF^{35}$ interact with each other and delineate the regions of both proteins that mediate this interaction. Using anti-peptide antibodies against $U2AF^{35}$, we show that the protein has the intracellular distribution characteristic of $U2AF^{65}$. Both $U2AF^{65}$ and $U2AF^{35}$ are concentrated in a small number of nuclear foci corresponding to coiled bodies, subnuclear organelles first identified by light microscopy in 1903.**

In vitro experiments demonstrate that pre-mRNA splicing begins with the assembly of a multicomponent complex on the pre-mRNA (reviewed in refs. 1–4). This complex, the spliceosome, contains two types of factors: small nuclear ribonucleoprotein particles (snRNPs) and proteins. These components assemble on the RNA according to an ordered pathway. Several of these steps require ATP hydrolysis, the first of which is U2 snRNP binding.

Binding of U2 snRNP to the pre-mRNA branch site requires, in addition to U2 snRNP itself, U1 snRNP (5) and at least three protein factors: SF1, SF3 (6), and U2 snRNP auxiliary factor (U2AF; refs. 7 and 8). Of these protein factors, U2AF has been most extensively characterized.

Purified HeLa cell U2AF comprises two polypeptides, 65 and 35 kDa (8, 9). Our previous studies (8–10) have shown that the U2AF large subunit ($U2AF^{65}$) was essential for *in vitro* splicing of an adenovirus major late and a human β-globin pre-mRNA, but that the U2AF small subunit ($U2AF^{35}$) was dispensable. However, $U2AF^{35}$ may be required for splicing other pre-mRNAs (e.g., those that are inefficiently spliced), regulating selection of splice sites, or providing some intracellular function (e.g., subcellular localization). $U2AF^{35}$ is evolutionarily conserved (9) and copurifies with $U2AF^{65}$ (8). In this report, we describe the isolation of a cDNA clone encoding $U2AF^{35}$.‡

METHODS

Membrane-Bound Protein Binding Assay. Ten microliters of HeLa cell nuclear extract, U2AF-depleted nuclear extract, or purified U2AF was separated on a SDS/12.5% polyacrylamide gel and then transferred to poly(vinylidene difluoride) membranes (Millipore) by electroblotting. The membrane was blocked for 1 h with 5% bovine serum albumin (BSA) in 20 mM Hepes·KOH, pH 7.9/3 mM $MgCl_2$/0.1 mM EDTA/0.1% Tween 20/10% (vol/vol) glycerol/1 mM dithiothreitol/100 mM to 1 M KCl. The membrane was incubated overnight with ^{35}S-labeled *in vitro* translated $U2AF^{65}$ or $U2AF^{35}$ (20 μl) in 10 ml of blocking buffer and was then washed three times with the same buffer without BSA. The membrane was then dried and exposed to x-ray film.

Intracellular Localization Experiments. The anti-peptide antibody to $U2AF^{35}$ was described by Zamore and Green (9), where it was referred to as anti-pepC. The affinity-purified antibody (1.5 mg/ml) was injected into the nucleus of living HeLa cells by an AIS microinjection system (11). Cells were incubated for 1 h at 37°C, washed with phosphate-buffered saline (PBS), transferred to ice, extracted with 0.5% Triton X-100 in CSK buffer for 3 min, and fixed for 10 min with 3.7% paraformaldehyde in CSK buffer as described (12). After fixation, the cells were washed with 0.5% Tween 20 in PBS and then sequentially incubated with anti-p80 coilin patient antiserum (kindly provided by E. H. Tan, Scripps Clinic) diluted 1:200 in PBS and Texas Red-conjugated goat anti-human IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Dianova, Hamburg, F.R.G.). Staining of fixed cells and confocal fluorescence microscopy were as described (12, 13).

RESULTS AND DISCUSSION

Isolation of a cDNA Clone for $U2AF^{35}$. To isolate a cDNA encoding the 35-kDa U2AF polypeptide, purified HeLa cell U2AF (8, 9) was separated by electrophoresis and transferred to a nitrocellulose membrane. The region of the membrane corresponding to the 35-kDa subunit was isolated and digested with trypsin. The resulting peptides were resolved by high-pressure liquid chromatography, and the sequences of three were determined (boxed). The sequences of two peptides permitted the design of degenerate primers that allowed us to isolate by PCR the nondegenerate DNA encoding each peptide. The two PCR products were cloned and sequenced. PCR primers were next designed to permit the isolation of the DNA between the two smaller DNA sequences. The product of this PCR was again cloned and sequenced. Finally, a portion of the sequence was used to screen ≈5 × 10^6 plaques of a λgt10 human fetal brain cDNA library. Twenty-five positive clones were identified and purified and two of these were subcloned and sequenced. Both encoded all three peptides identified by protein microsequencing.

Fig. 1 shows the sequence of a 931-base-pair cDNA for $U2AF^{35}$. The cDNA contains a 240-amino acid open reading

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Abbreviations: snRNP, small nuclear ribonucleoprotein; U2AF, U2 snRNP auxiliary factor.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96982).

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-31                                          +1
CGTCG ACGGC AGCGG CGGCG GCGGG TGGGA A ATG GCG GAG TAT CTG GCC TCC ATC      24
                                      M  A  E  Y  L  A  S  I          8

TTC GGC ACC GAG AAA GAC AAA GTC AAC TGT TCA TTT TAT TTC AAA ATT GGA GCA    78
 F  G  T  E  K  D  K  V  N  C  S  F  Y  F  K  I  G  A                  26

TGT CGT CAT GGA GAC AGG TGC TCT CGG TTG CAC AAT AAA CCG ACG TTT AGC CAG   132
C  R  H  G  D  R  C  S  R  L  H  N  K  P  T  F  S  Q                  44

ACC ATT GCC CTC TTG AAC ATT TAC CGT AAC CCT CAA AAC TCT TCC CAG TCT GCT   186
 T  I  A  L  L  N  I  Y  R  N  P  Q  N  S  S  Q  S  A                  62

GAC GGT TTG CGC TGT GCC GTG AGC GAT GTG GAG ATG CAG GAA CAC TAT GAT GAG   240
 D  G  L  R  C [A  V  S  D  V  E  M  Q  E  H  Y  D  E]                  80
                                                pep 79

TTT TTT GAG GAG GTT TTT ACA GAA ATG GAG GAG AAG TAT GGG GAA GTA GAG GAG   294
..F....F  E  E  V  F  T  E  M] E  E  K  Y  G  E  V  E  E                98

ATG AAC GTC TGT GAC AAC CTG GGA GAC CAC CTG GTG GGG AAC GTG TAC GTC AAG   348
 M  N  V  C  D  N  L  G  D  H  L  V  G  N  V  Y  V  K                 116

TTT CGC CGT GAG GAA GAT GCG GAA AAG GCT GTG ATT GAC TTG AAT AAC CGT TGG   402
 F  R  R  E  E  D  A  E  K [A  V  I  D  L  N  N  R] W                 132
                                      pep 18

TTT AAT GGA CAG CCG ATC CAC GCC GAG CTG TCA CCC GTG ACG GAC TTC AGA GAA   456
 F  N  G  Q  P  I  H  A  E  L  S  P  V  T  D  F  R  E                 150
                   pep 54

GCC TGC TGC CGT CAG TAT GAG ATG GGA GAA TGC ACA CGA GGC GGC TTC TGC AAC   510
 A  C  C  R  Q  Y  E  M  G  E  C  T  R  G  G  F  C  N                 168

TTC ATG CAT TTG AAG CCC ATT TCC AGA GAG CTG CGG CGG GAG CTG TAT GGC CGC   564
 F  M  H  L  K  P  I  S  R  E  L  R  R  E  L  Y  G  R                 186

CGT CGC AAG AAG CAT AGA TCA AGA TCC CGA TCC CGG GAG CGT CGT TCT CGG TCT   618
 R  R  K  K  H  R  S  R  S  R  S  R  E  R  R  S  R  S                 204

AGA GAC CGT GGT CGT GGC GGT GGC GGT GGC GGT GGT GGA GGT GGC GGC GGA CGG   672
 R  D  R  G  R  G  G  G  G  G  G  G  G  G  G  G  G  R                 222

GAG CGT GAC AGG AGG CGG TCG AGA GAT CGT GAA AGA TCT GGG CGA TTC TGA G     726
 E  R  D  R  R  R  S  R  D  R  E  R  S  G  R  F  *                    238

CCATG CCATT TTTAC CTTAT GTCTG CTAGA AAGTG TTGTA GTTGA TTGAC CAAAC CAGTT   786
CATAA GGGGA ATTTT TTAAA AAACA ACAAA AAAAA AACAT ACAAA GATGG GTTTC TGAAT   846
AAAAA TTTGT AGTGA TAACA GTAAA AAAAA AAAAA AAAAA AAAAA AAAAA AAAGG AA      903
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FIG. 1. Isolation of a cDNA encoding the 35-kDa U2AF subunit. The sequence of a U2AF^{35} cDNA is shown and the deduced protein sequence is indicated in one-letter amino acid code. Peptides determined by microsequencing tryptic peptides are boxed. The peptide used to raise the anti-U2AF^{35} antibody (pepC; ref. 2) is underlined. Unless otherwise stated, the procedures for the isolation and sequencing of cDNA clones were as described (10).

frame that would encode a protein with a predicted molecular mass of 34 kDa. The putative initiator methionine codon is flanked by codons that contain two highly conserved G residues (positions +4 and −3) that are typical of strong translation initiation sites (14). Furthermore, the region preceding this AUG contains seven CpG dinucleotides; 5′ untranslated sequences are typically CpG-rich (15).

To determine the size of the mRNA encoding U2AF^{35}, we prepared a Northern blot with HeLa cell total RNA using ^{32}P-labeled cloned cDNA insert as a probe (Fig. 2). The probe detected a single ≈950-nucleotide mRNA, which is of sufficient size to encode U2AF^{35}. *In vitro* translation of RNA containing the U2AF^{35} coding sequence gave rise to a 35-kDa polypeptide that comigrated with purified HeLa U2AF^{35} (Fig. 3*A*; data not shown). Taken together, these observations indicate that the U2AF^{35} cDNA clone is complete.

Sequence Features of U2AF^{35}. The two most striking features of the predicted amino acid sequence of U2AF^{35} (Fig. 1) are an arginine, serine (RS)-rich region (residues 188–239) and a run of 12 consecutive glycine residues (residues 232–223). RS-rich regions constitute a motif found in a growing number of splicing factors including the U1 snRNP-specific 70-kDa protein (16–19), ASF/SF2 (20, 21), and U2AF^{65} (10), as well as the *Drosophila* splicing regulators transformer (22), transformer-2 (23, 24), suppressor-of-sable (25), and suppressor-of-white apricot (26). The 12-residue glycine run of

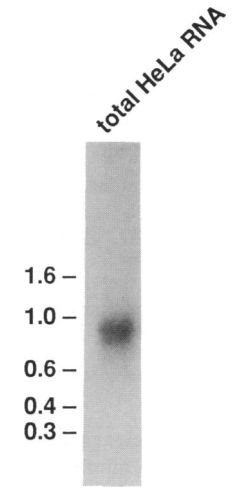

FIG. 2. Northern blot analysis. Northern blotting was performed as described (10) using total HeLa cytoplasmic RNA. The ^{32}P-labeled DNA probe contained the entire U2AF^{35} cDNA. Size markers (kb) are indicated on the left.

U2AF^{35} is reminiscent of the nine consecutive glycines present in the mammalian splicing factor ASF/SF2 (20, 21). Interestingly, in U2AF^{35}, the glycine stretch interrupts the RS-rich region. U2AF^{35} does not contain a consensus RNP CS motif (27) or identifiable helicase or nucleotide binding motifs.

***In Vitro* Interaction Between U2AF^{65} and U2AF^{35}.** U2AF^{35} was originally identified because of its copurification with U2AF^{65} (8). The two U2AF subunits cosediment with a native molecular mass consistent with an ≈100-kDa heterodimer (8). To confirm that the two isolated U2AF subunits could interact with one another, we performed a membrane-immobilized protein binding assay. Crude HeLa cell nuclear extract, U2AF-depleted nuclear extract, or purified HeLa U2AF was separated on a SDS/polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane. The membrane was probed with ^{35}S-labeled, *in vitro* translated U2AF^{35} or U2AF^{65}. Binding of the *in vitro* translated polypeptide to membrane-bound proteins was detected by autoradiography.

Fig. 3 *B* and *C* shows that both *in vitro* translated U2AF^{35} and U2AF^{65} reconstituted the U2AF heterodimer by binding specifically to the other membrane-bound U2AF subunit. Fig. 3*B* shows that U2AF^{35} bound to a 65-kDa polypeptide in the nuclear extract that comigrated with the 65-kDa component of HeLa U2AF. This 65-kDa polypeptide was not detected in an extract chromatographically depleted of U2AF (ΔU2AF-NE). A prominent ≈35-kDa doublet was also detected, but it is unrelated to U2AF because it is present in the ΔU2AF-NE. The interaction between U2AF^{35} and this protein is likely nonspecific, because it is unaffected by mutations in U2AF^{35} (see below).

Fig. 3*C* shows that U2AF^{65} bound a 35-kDa polypeptide present in the crude nuclear extract and in a purified HeLa U2AF preparation. This 35-kDa polypeptide comigrated with the 35-kDa component of purified HeLa U2AF and was absent from the U2AF-depleted nuclear extract (ΔU2AF-NE). The interaction between the two U2AF subunits was stable even in 1 M KCl, suggesting that hydrophobic rather than ionic interactions are primarily involved.

To map the region of each U2AF polypeptide involved in this protein–protein interaction, we probed membrane-bound nuclear extract with a series of *in vitro* translated U2AF derivatives. The *in vitro* translation products are shown in Fig. 3*A* and the protein binding assays are shown in Fig. 3 *B* and *C*. Fig. 3*B* shows that N-terminal deletion to residue 46

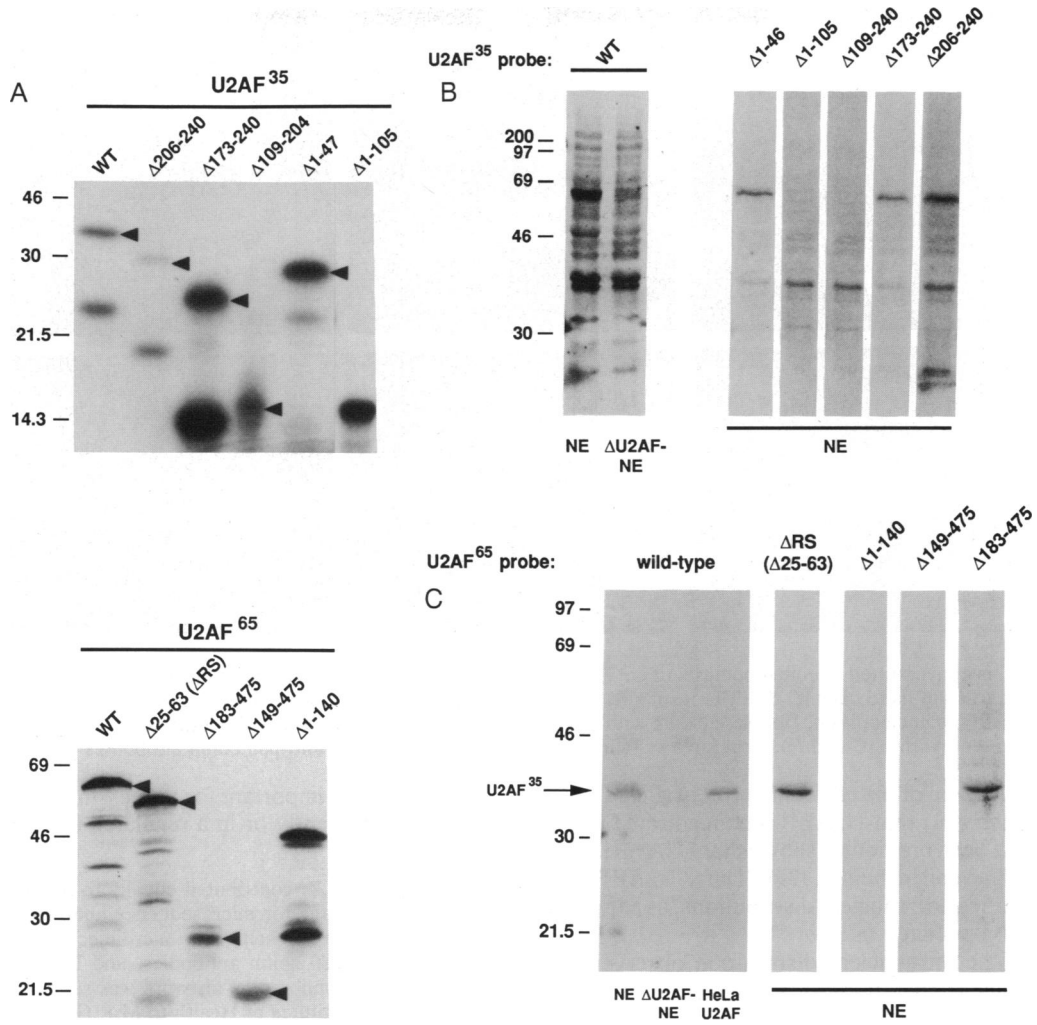

FIG. 3. Mapping the interaction surfaces of the two U2AF subunits. (*A*) Protein derivatives used in this study. The U2AF^{65} and U2AF^{35} coding sequences were fused to the human β-globin 5′ untranslated sequence. RNA was transcribed from these plasmids with SP6 RNA polymerase and was translated in a wheat germ extract. Protein derivatives were created by linearizing the plasmid at appropriate restriction sites. The deletion mutant ΔRS has been described (10). The full-length *in vitro* translation products are indicated by arrows. Size standards (kDa) are indicated on the left. (*B*) U2AF^{35} binds U2AF^{65}. The protein preparation used is indicated below and the ^{35}S-labeled *in vitro* translated derivative is indicated above. Size standards (kDa) are indicated on the left. The position of U2AF^{65} is shown. (*C*) U2AF^{65} binds U2AF^{35}. Other details are the same as in *B*. The position of U2AF^{35} is shown. WT, wild type; NE, nuclear extract.

did not affect the ability of U2AF^{35} to bind U2AF^{65}, but further deletion to residue 105 eliminated the interaction. C-terminal deletion of U2AF^{35} to residue 173 had no effect, but further deletion to residue 109 abolished interaction with U2AF^{65}. Thus, a region between amino acids 47 and 172 of U2AF^{35} is involved in binding to U2AF^{65}. This region does not include the RS motif or the glycine run.

A similar examination of U2AF^{65} sequences (Fig. 3*C*) showed that neither its N-terminal RS motif (amino acids 25–63) nor an intact C-terminal sequence-specific RNA binding domain (amino acids 151–462) was required for interaction with U2AF^{35}. An in-frame deletion of the N-terminal RS motif (ΔRS) bound U2AF^{35} normally, whereas deletion of the N-terminal 140 amino acids eliminated the interaction with U2AF^{35}. Deletion of amino acids C-terminal to residue 183 did not affect binding but further deletion to residue 149 disrupted the interaction. Thus, a region between amino acids 64 and 182 of U2AF^{65} is involved in binding to U2AF^{35}.

Intracellular Localization of U2AF^{35}. The strong, specific *in vitro* interaction between the two U2AF subunits prompted us to ask whether the two proteins colocalize *in vivo*. We have previously reported that U2AF^{65} is distributed throughout the nucleoplasm and concentrated in a small number of nuclear foci (9). This same intranuclear distribution has been observed for U1 snRNA (13). U2, U4/6, and U5 snRNAs are highly concentrated in the foci (13) and can only be detected in the nucleoplasm upon long times of hybridization (28) or following extraction of fixed cells with SDS (29).

It has been recently shown that these snRNA-containing foci correspond to previously identified nuclear structures designated coiled bodies (29). Autoantibodies to the protein p80 coilin, a constituent of the coiled body (30), provide a convenient marker for these intranuclear structures. We used double-label immunofluorescence to determine the intranuclear distribution of U2AF^{35} relative to that of p80 coilin. In these experiments, an affinity-purified, anti-peptide antibody against U2AF^{35} (described in ref. 9) was either microinjected into the cell nucleus before fixation (Fig. 4 *A–C*) or was used to stain fixed cells (Fig. 4 *D–F*). Fig. 4 *A* and *D* shows that like U2AF^{65} (9), U2AF^{35} was concentrated in a small number of foci (arrows) and was diffusely distributed throughout the nucleoplasm excluding nucleoli. The staining observed with the anti-U2AF^{35} antibody was eliminated by addition of the same U2AF^{35} peptide used to derive the antibody (pepC; ref. 9) but not by an equal amount of an unrelated peptide (data not shown). These U2AF^{35}-containing foci correspond to the

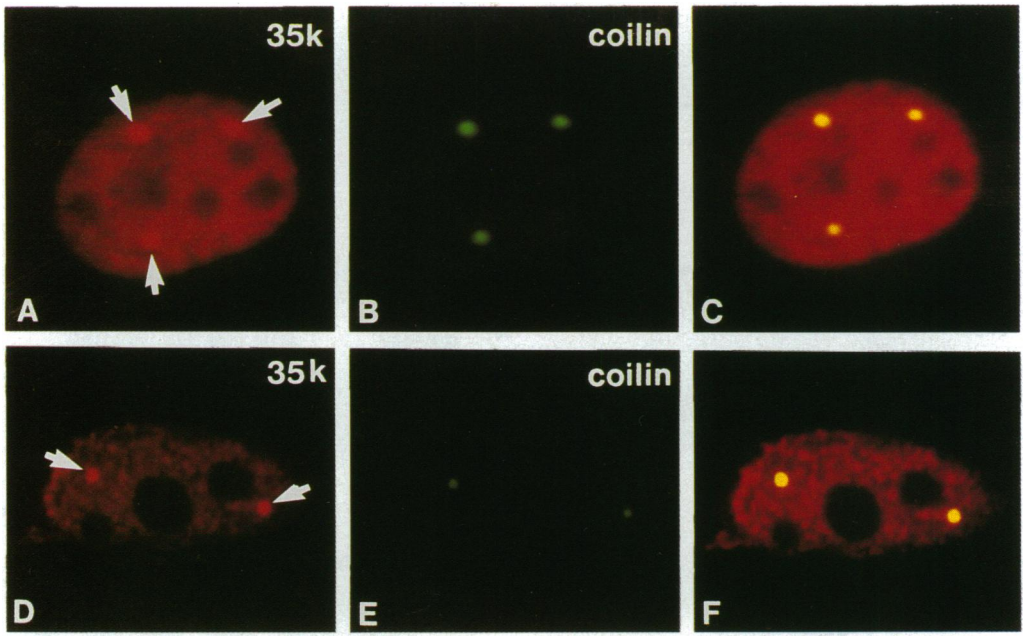

FIG. 4. U2AF^{35} is concentrated in coiled bodies. An anti-peptide against U2AF^{35} was either microinjected into the nucleus of living HeLa cells (*A–C*) or used to stain fixed cells (*D–F*). The results (*A* and *D*) show that U2AF^{35} was concentrated in discrete foci (arrows) as well as distributed throughout the nucleoplasm. Double-labeling with anti-p80 coilin antibody (*B*, *C*, *E*, and *F*) demonstrates that these foci correspond to coiled bodies. In the overlay (*C* and *F*), structures staining with both anti-U2AF^{35} and anti-p80 coilin antibodies appear yellow.

intranuclear localization of the p80 coilin protein (Fig. 4 *B*, *C*, *E*, and *F*) demonstrating that U2AF^{35} is concentrated in the coiled body. It has been previously shown that U2AF^{65} is also concentrated in the coiled bodies (29). Thus, U2AF^{35} and U2AF^{65} have identical intranuclear distributions, as expected if they are stably associated *in vivo*.

In contrast to the intranuclear distribution observed for U2AF and the spliceosomal snRNAs, some snRNP structural proteins and the splicing factor SC-35 are concentrated in a larger number of discrete "speckles" (reviewed in ref. 1). Recently, it was proposed that the RS motif targets proteins to these speckled nuclear subregions (31). Our studies with U2AF demonstrate that the RS motif is not a dominant subnuclear targeting signal: both U2AF^{35} (this report) and U2AF^{65} (10) contain RS motifs, but neither has a speckled distribution. Instead, both U2AF^{35} and U2AF^{65} are distributed throughout the nucleoplasm and concentrated in coiled bodies. Our studies do not exclude, however, that the RS motif can affect nuclear localization by, for example, interacting with spliceosomal components, which themselves are distributed nonrandomly.

Several factors implicated in mammalian pre-mRNA splicing are ≈35 kDa, including U2AF^{35}, ASF/SF2 (20, 21), and SC-35 (32). The relationship among these three factors is unclear and has been a source of confusion. The sequence of U2AF^{35} (Fig. 1) reveals that it is distinct from both ASF/SF2 (20, 21) and SC-35 (33, 34).

In light of the strong, specific interaction between the two U2AF subunits, what role might U2AF^{35} play in pre-mRNA splicing? We have recently shown that the RS motif of U2AF^{65} is required for splicing *in vitro* (10). U2AF^{65} binds a specific sequence element, the polypyrimidine tract, thereby bringing the U2AF^{65} RS motif to the pre-mRNA. Because U2AF^{35} interacts with U2AF^{65}, its RS motif will also be brought to the pre-mRNA following binding of U2AF^{65}. We speculate that the RS motifs of U2AF^{35} and U2AF^{65} cooperate with one another to facilitate splicing by, for example, contacting the same component during spliceosome assembly. Such cooperative action of the two U2AF subunits may be particularly important for processing pre-mRNAs that are spliced inefficiently or in a regulated fashion.

M.Z. and P.D.Z. contributed equally to the work reported here. We thank W. Lane for microsequence analysis, Drs. R. Peperkok and W. Ansorge for help with microinjection, Prof. Eng Tan for providing anti-p80 coilin antibodies, and L. Kunkel for the λgt10 human fetal brain library. This work was supported by a grant from the National Institutes of Health to M.R.G.

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