U2AF⁶⁵ recruits a novel human DEAD box protein required for the U2 snRNP–branchpoint interaction

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Splicing of mRNA precursors (pre-mRNAs) comprises a series of ATP-dependent steps, the first of which is the stable binding of U2 snRNP at the pre-mRNA branchpoint. The basis of ATP use for the interaction between U2 snRNP and the branchpoint is unclear, and, in particular, none of the known mammalian factors required for this step have the sequence characteristics of proteins that hydrolyze ATP. Entry of U2 snRNP into the spliceosome is initiated by interaction of the essential splicing factor U2AF⁶⁵ with the pre-mRNA polypyrimidine tract. In this report we identify a new region of U2AF⁶⁵ required for function, and use this information to clone a human 56-kD U2AF⁶⁵ associated protein (UAP56). We show that UAP56 is an essential splicing factor, which is recruited to the pre-mRNA dependent on U2AF⁶⁵, and is required for the U2 snRNP–branchpoint interaction. The sequence of UAP56 indicates it is a member of the DEAD box family of RNA-dependent ATPases, which mediate ATP hydrolysis during several steps of yeast pre-mRNA splicing. Our results reveal a new function of U2AF⁶⁵: to position a DEAD box protein required for U2 snRNP binding at the pre-mRNA branchpoint region.

[Key Words: U2 snRNP; branchpoint; U2AF⁶⁵; DEAD box protein; pre-mRNA splicing]

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Nuclear pre-mRNA splicing is catalyzed by a dynamic ribonucleoprotein, the spliceosome, whose formation involves the stepwise assembly of four (U1, U2, U5, and U4/U6) small ribonucleoproteins (snRNPs), as well as many non-snRNP protein splicing factors (for review, see Green 1991; Bennett et al. 1992; Rymond and Rosbash 1992; Moore et al. 1993; Madhani and Guthrie 1994; Nilsen 1994; Kramer 1996). Several of the steps involved in spliceosome assembly require ATP hydrolysis, the first of which is the stable binding of U2 snRNP at the pre-mRNA branchpoint.

In the yeast *Saccharomyces cerevisiae* genetic screens have identified >40 proteins involved in splicing (for review, see Guthrie 1991; Ruby and Abelson 1991; Rymond and Rosbash 1992; Madhani and Guthrie 1994; Kramer 1996). Among these, six [PRP2, PRP5, PRP16, PRP22, PRP28 (PRP stands for pre-mRNA processing mutant) and Snu246p] are members of the DEAD box family of RNA-dependent ATPases (for review, see Schmid and Linder 1992; Fuller-Pace 1994). The found-

ing member of this family is eIF-4A, a eukaryotic translation initiation factor that is required for mRNA binding to ribosomes. EIF-4A exhibits single-stranded RNA-dependent ATPase activity, and in combination with a second initiation factor, eIF-4B, also possesses duplex RNA helicase activity. An attractive idea is that yeast DEAD box proteins are required to unwind particular RNA hybrids that are formed during spliceosome assembly and splicing. To date, however, none of the DEAD box proteins implicated in splicing have demonstrable RNA helicase activity, although several have been shown to possess RNA-dependent ATPase activity (Schwer and Guthrie 1991; Kim and Lin 1993; Xu et al. 1996).

Biochemical experiments have shown that stable binding of mammalian U2 snRNP at the pre-mRNA branchpoint requires several auxiliary factors, including U2AF, SF1, SF3a, SF3b, and U1 snRNP (for review, see Moore et al. 1993; Hodges and Beggs 1994; Reed 1996). U2 snRNP-binding requires ATP hydrolysis and it is therefore puzzling that none of the forementioned mammalian splicing factors have the sequence motifs of proteins that use ATP. In yeast, the DEAD box protein PRP5 is an essential splicing factor specifically required for entry of U2 snRNP into the spliceosome (Ruby et al. 1993; Wells and Ares 1994). The strong conservation between the splicing mechanisms and machineries in yeast and humans

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(for review, see Rymond and Rosbash 1992; Moore et al. 1993; Hodges and Beggs 1994; Madhani and Guthrie 1994; Nilsen 1994; Kramer 1996; Reed 1996) implies the existence of a DEAD box protein also required for the U2 snRNP-branchpoint interaction in mammalian cells.

U2AF⁶⁵, an essential mammalian splicing factor, initiates U2 snRNP-binding by interacting through its carboxy-terminal RNA-binding domain with the polypyrimidine (Py) tract adjacent to the 3' splice site (Zamore et al. 1992). U2AF⁶⁵ also contains an amino-terminal arginine/serine-rich (RS) domain, which is essential for function and separated from its RNA-binding domain by a 56-amino-acid linker region (amino acids 95–151) [see Fig. 1A]. Recent studies have shown that the U2AF⁶⁵ RS domain functions, at least in part, by contacting the branchpoint and facilitating annealing with U2 snRNA (Valcárcel et al. 1996).

In this report we identify a new region of U2AF⁶⁵ required for function and use this information to clone a human 56-kD U2AF⁶⁵-associated protein (UAP56), which is a member of the DEAD box family of RNA-dependent ATPases. Our data suggest a mechanism for targeting a specific DEAD box splicing factor to the pre-mRNA branchpoint region.

Figure 1. A third region of U2AF⁶⁵ required for function. (A) Schematic representation of U2AF⁶⁵-Sxl derivatives. (RS) Arginine/serine-rich domain; (RNP-CS) ribonucleoprotein consensus motif; (Sxl RBD) RNA-binding domain of Sxl (amino acids 96–323). (B) Splicing activity of U2AF⁶⁵-Sxl fusion proteins. In vitro splicing reactions were performed either using nuclear extract (NE) (lane 1) or U2AF-depleted nuclear extract (ΔU2AF NE) supplemented with the indicated protein at a final concentration of 50 ng/μl (lanes 2–7). Accurately spliced mRNA was detected by primer extension using a ³²P-labeled DNA primer complementary to the spliced junction as described previously (Valcárcel et al. 1993).

Results

A third region of U2AF⁶⁵ required for function

We have shown previously that the *Drosophila* Sex-lethal protein (Sxl), a splicing repressor, can be endowed with U2AF⁶⁵ activity by fusion of the amino-terminal 228 amino acids of U2AF⁶⁵ (Valcárcel et al. 1993; Fig. 1B, lane 5). To delineate in further detail the portion of U2AF⁶⁵ required to confer activity we constructed and analyzed several additional U2AF⁶⁵-Sxl fusion proteins (Fig. 1A). In this experiment and those described below, U2AF⁶⁵ derivatives were expressed in and purified from *Escherichia coli* and their activities analyzed following addition to a HeLa nuclear extract specifically depleted of U2AF (Zamore and Green 1991; Zamore et al. 1992; Valcárcel et al. 1993, 1996). Figure 1B shows unexpectedly that fusion of a minimal RS domain (amino acids 1–55) to the Sxl RNA-binding domain was not sufficient to confer splicing activity (lane 6), implicating residues 56–233 in U2AF⁶⁵ function. Thus, these results and those described below indicate that U2AF⁶⁵ contains a third region required for function.

To confirm and extend these results, we constructed three in-frame deletion mutants (U2AF⁶⁵Δ95–138, U2AF⁶⁵Δ95–161, and U2AF⁶⁵Δ95–183; see Fig. 2A) and analyzed their abilities to support in vitro splicing. These three U2AF⁶⁵ in-frame deletion mutants bound to the pre-mRNA Py tract with an affinity and specificity comparable to that of wild-type U2AF⁶⁵ (data not shown). Figure 2B shows that U2AF⁶⁵Δ95–138 supported splicing (lanes 6–8), whereas U2AF⁶⁵Δ95–161 (lanes 9–11) and U2AF⁶⁵Δ95–183 (lanes 12–14) did not. Taken together, these data indicate that amino acids 138–183 are within a third region of U2AF⁶⁵ required for activity.

Isolation of a U2AF⁶⁵ interacting protein

It seemed most likely that this third U2AF⁶⁵ region would function by interacting with another essential splicing factor. U2AF is a heterodimer comprising 65-kD and 35-kD subunits, and the U2AF³⁵ interaction site is within the U2AF⁶⁵ linker region (Zhang et al. 1992). Thus, one explanation for the inactivity of the two U2AF⁶⁵ deletion mutants was a failure to interact with U2AF³⁵. To test this possibility we used a yeast two-hybrid assay to analyze the interaction between U2AF³⁵ and the U2AF⁶⁵ derivatives. As summarized in Table 1, U2AF³⁵ interacted with wild-type U2AF⁶⁵ but not with U2AF⁶⁵Δ95–138, which fully supported pre-mRNA splicing (Fig. 2B). Therefore, the splicing defect of the U2AF⁶⁵Δ95–161 and U2AF⁶⁵Δ95–183 mutants cannot be explained by an inability to interact with U2AF³⁵.

To identify the splicing factor hypothesized to interact with U2AF⁶⁵ amino acids 138–183, we screened a HeLa cDNA library in a yeast two-hybrid assay (summarized in Table 1). Screening of ~60 million yeast colonies with a LexA-U2AF⁶⁵ fusion protein yielded 69 positives. Interaction specificity was determined by testing whether each candidate bound to LexA alone, LexA-U2AF³⁵, or an irrelevant LexA fusion protein (LexA-Rex). This ex-

Figure 2. Analysis of U2AF⁶⁵ in-frame deletion mutants. Schematic representation (A; see Fig. 1 legend) and splicing activity (B) of U2AF⁶⁵ in-frame deletion mutants. Splicing in either nuclear (lane 2) or U2AF-depleted nuclear extract supplemented with the indicated proteins, at a final concentration of 25, 50, or 100 ng/μl (lanes 3–15) was analyzed as in Fig. 1B. (F.L.) Full-length U2AF⁶⁵.

periment eliminated 14 clones, leaving 55 candidates that encoded polypeptides specifically interacting with U2AF⁶⁵. Sequence analysis revealed that 35 of these cDNAs encoded U2AF³⁵. For the remaining 20 clones, the U2AF⁶⁵ interaction site was determined. Table 1 shows that none of the clones encoded polypeptides that interacted with the U2AF⁶⁵ RS domain, consistent with the notion that this region contacts the pre-mRNA branchpoint and not another splicing factor (Valcárcel et al. 1996). Two clones, which were independent cDNA isolates derived from the same gene, specifically interacted with U2AF⁶⁵ amino acids 138–183, which were implicated in splicing activity (Fig. 2). We shall refer to the protein encoded by this cDNA as UAP56 (56-kD U2AF associated protein).

Figure 3 shows the sequence of full-length human UAP56, which contains a 428-amino-acid open reading frame. Databank searches identified several apparent UAP56 homologs, originally isolated as expressed sequence tags or for other reasons unrelated to splicing. Importantly, and as discussed in greater detail below, UAP56 contains sequence motifs characteristic of the DEAD box RNA helicase family of proteins (Schmid and Linder 1992; Pause et al. 1993).

In vitro interaction between U2AF⁶⁵ and UAP56

To determine whether U2AF⁶⁵ and UAP56 interact with one another in a HeLa nuclear extract, we performed a coimmunoprecipitation experiment (Fig. 4). First, we derived a polyclonal α-UAP56 antibody, which detected a single, 56-kD polypeptide in HeLa nuclear extract (lane

1). UAP56 immunoprecipitates were assayed for the presence of U2AF⁶⁵ by immunoblotting. These data indicate that α-UAP56 immunoprecipitates contained U2AF⁶⁵ (lane 3), whereas a control immunoprecipitate formed with preimmune serum did not (lane 2). Following RNase treatment of the nuclear extract, U2AF⁶⁵ and UAP56 were still coimmunoprecipitated (data not shown), indicating that the interaction was not mediated by RNA. The *in vitro* interaction between U2AF⁶⁵ and UAP56 provides further evidence for the *in vivo* interaction detected in the yeast two-hybrid assay.

UAP56 is an essential splicing factor

Several experiments were performed to assess whether UAP56 was an essential pre-mRNA splicing factor. We raised an α-UAP56 antibody and used this to prepare nuclear extracts specifically depleted of UAP56. Figure 5A shows that immunodepletion with the α-UAP56 antibody reduced the concentration of UAP56 in a HeLa nuclear extract >98% (cf. lanes 1 and 2) and rendered the extract unable to support splicing (Fig. 5C, lane 3). UAP56 was initially purified from a HeLa cell nuclear extract using a chromatographic scheme involving a final stringent immunopurification step (see Materials and Methods). SDS-PAGE and Coomassie blue staining showed that the purified material comprised a major 56-kD polypeptide (Fig. 5B, lane 2), which immunoblotting confirmed as UAP56 (Fig. 5D, lane 8). Addition of the purified UAP56 to the immunodepleted extract rescued splicing (Fig. 5C, lane 5). The immunoblot of Figure 5D

Table 1. Summary of yeast two-hybrid data

Yeast colonies screened	60 × 10 ⁶		
Clones positive for binding	69		
U2AF ⁶⁵ -specific binding	55		
	U2AF ³⁵	UAP56	All others
	35	2	18
U2AF ⁶⁵ derivative	Yeast two-hybrid interaction		
Full length	+	+	+
U2AF ⁶⁵ Δ1–64	+	+	+
U2AF ⁶⁵ Δ95–475	–	–	–
U2AF ⁶⁵ Δ234–475	+	+	–
U2AF ⁶⁵ Δ1–228	–	–	+
U2AF ⁶⁵ Δ95–138	–	+	N.D.
U2AF ⁶⁵ Δ95–161	–	–	N.D.
U2AF ⁶⁵ Δ95–183	–	–	N.D.

Full-length U2AF⁶⁵ or U2AF⁶⁵ derivatives were fused in-frame to LexA (1–202) in the vector pEG202 (Guyris et al. 1993). Colonies were selected based on galactose-dependent growth on Leu[–] medium and galactose-dependent blue staining on X-gal-containing medium. (+) Growth on Leu[–] medium and blue staining on X-gal-containing medium; (–) no growth on Leu[–] medium and colonies white on X-gal-containing medium. (N.D.) Not determined.

Figure 3. Amino acid sequence of human UAP56 and homologs. The clones obtained in the two-hybrid screen that specifically interacted with U2AF⁶⁵ amino acids 95–151 were characterized, and their sequences aligned to homologs from other species found in database searches using the Pileup program of the GCG sequence analysis software package. (–) Amino acid identity; (*) gaps. The seven consensus motifs characteristic of DEAD box RNA helicases [Schmid and Linder 1992; Pause et al. 1993] are indicated by shaded boxes. GenBank accession nos.: (*Sus scrofa*) Z34846; (*Rattus norvegicus*) M75168; (*Drosophila melanogaster*) X79802; (*Caenorhabditis elegans*) U08108; (*Homo sapiens*) Z37166.

indicates that U2AF⁶⁵ was present at relatively high concentrations in the UAP56-depleted extract (lane 3), and was undetectable in the purified UAP56 preparation (lane 4). Moreover, addition of glutathione S-transferase (GST)–U2AF⁶⁵, which restored splicing to a U2AF⁶⁵-depleted extract (e.g., Valcárcel et al. 1996), had no effect on the UAP56-depleted extract (data not shown). Thus, the splicing complementation results of Figure 5C cannot be explained by codepletion and readdition of U2AF⁶⁵. To verify this conclusion, human UAP56 was purified from insect cells infected with recombinant baculovirus. Figure 5E shows that following addition to the UAP56-depleted nuclear extract baculovirus-derived UAP56 restored splicing in a dose-dependent manner. Taken to-

gether, these results indicate that UAP56 is an essential splicing factor.

UAP56 is a spliceosomal component

The stable association between U2AF⁶⁵ and UAP56 suggested that UAP56 would be recruited to the spliceosome in a U2AF⁶⁵-dependent manner. To test this possibility a series of biotinylated RNAs were used to affinity-purify RNA–protein complexes, which were then analyzed for the presence of UAP56 by immunoblotting. Figure 6 shows that UAP56 was stably associated with wild-type pre-mRNA (lane 1) and a 5' splice site deletion mutant that could form only complex A (lane 2). In contrast, UAP56 was not stably associated with a pre-mRNA lacking a Py tract, the U2AF⁶⁵ binding site (lane 3), or with a wild-type pre-mRNA following incubation in a nuclear extract specifically depleted of U2AF⁶⁵ (lane 4). In conjunction with the results described above, these data indicate that UAP56 is a spliceosomal component that is recruited to the pre-mRNA through interaction with Py tract-bound U2AF⁶⁵.

UAP56 is required for the U2 snRNP–branchpoint interaction

The association of UAP56 only with pre-mRNAs that can form complex A (Fig. 6), and the well-established requirement of U2AF⁶⁵ for the U2 snRNP–branchpoint interaction, suggested that UAP56 would also act during this step. To test this possibility, we first examined the ability of the three U2AF⁶⁵ in-frame deletion mutants (Fig. 2A) to support U2 snRNP-binding. For this experiment and that of Figure 7B we used an RNA substrate that contained a 5' splice site deletion and thus formed only complex A (also called complex A3'). Figure 7A shows that U2AF⁶⁵ derivatives containing the UAP56 interaction site (full-length and U2AF⁶⁵Δ95–138) supported U2 snRNP binding, whereas U2AF⁶⁵ derivatives

Figure 4. Coimmunoprecipitation of U2AF⁶⁵ and UAP56. Antiserum directed against UAP56 (lane 3) or preimmune serum (lane 2) were cross-linked to protein A beads and used for immunoprecipitation of HeLa nuclear extract. Bound proteins were eluted from the beads, electrophoresed on an SDS–polyacrylamide gel, and assayed for U2AF⁶⁵ by immunoblotting. (Lane 1) Immunoblot of HeLa nuclear extract with the α-UAP56 antiserum.

Figure 5. UAP56 is an essential splicing factor. (A) Immunodepletion of UAP56. HeLa nuclear extract was immunodepleted with either preimmune serum (lane 1) or the α -UAP56 serum (lane 2) and the resultant extracts analyzed for UAP56 by immunoblotting. (B) Immunopurification of UAP56. Immunopurified UAP56 was electrophoresed on an SDS-polyacrylamide gel and visualized by staining with Coomassie brilliant blue. (C) Biochemical complementation assay. Splicing reactions were carried out in the presence of nuclear extract (lane 1), preimmune antiserum-depleted nuclear extract (lane 2), or the UAP56-depleted nuclear extract (lanes 3–5). Immunopurified UAP56 was added to a final concentration of 2 (lane 4) or 5 ng/ μ l (lane 5). (D) Immunodetection of U2AF⁶⁵ and UAP56. U2AF⁶⁵ and UAP56 were detected by immunoblotting in HeLa nuclear extract (lanes 1,5), mock-depleted nuclear extract (lanes 2,6), UAP56-depleted nuclear extract (lanes 3,7) and immunopurified UAP56 (lanes 4,8). (E) Baculovirus-derived human UAP56 restores splicing to a UAP56-depleted nuclear extract. Splicing reactions were carried out in a UAP56-depleted nuclear extract following addition of baculovirus-derived His-UAP56 to a final concentration of 0, 5, 10, 20, or 50 ng/ μ l.

lacking the UAP56 interaction site (U2AF⁶⁵ Δ 95–161 and U2AF⁶⁵ Δ 95–183) did not.

To confirm the requirement of UAP56 for the U2 snRNP-branchpoint interaction, we performed a biochemical complementation experiment. Figure 7B shows that in a UAP56-depleted nuclear extract complex A formation was very inefficient (lane 9) and was greatly enhanced by addition of immunopurified HeLa UAP56 (lane 10). Taken together, these results indicate that UAP56 is an essential splicing factor, specifically required for the stable interaction between U2 snRNP and the pre-mRNA branchpoint.

Discussion

In this study we have identified a novel human DEAD-box protein, targeted to the pre-mRNA by U2AF⁶⁵ and required for the U2 snRNP-branchpoint interaction, the first ATP-dependent step in splicing. UAP56 has all the properties predicted for the human splicing factor that hydrolyzes ATP to mediate the stable addition of U2 snRNP onto the pre-mRNA branchpoint.

At least six yeast DEAD box proteins are known to act

at various steps during splicing (Rymond and Rosbash 1992; Madhani and Guthrie 1994; Kramer 1996). How these proteins are targeted to specific pre-mRNA signals at the appropriate time has not been elucidated. Our results provide a description of how a specific DEAD box protein is directed to a pre-mRNA splicing signal at a particular stage of the reaction.

Sequence features of UAP56

Figure 3 indicates that UAP56 contains all seven sequence motifs (boxed) characteristic of DEAD box proteins (Schmid and Linder 1992; Pause et al. 1993). Interestingly, UAP56 contains a DECD motif, conserved from *Caenorhabditis elegans* through humans, instead of the canonical DEAD/DEAH sequence. Yeast PRP5 is a DEAD box protein required for entry of U2 snRNP into the spliceosome (Ruby et al. 1993; Wells and Ares 1994). It is thus intriguing that human UAP56 and yeast PRP5 have an overall homology of 55% with 30% identity. However, similar to the relationship between yeast PRP22 and its human homolog, HRH1 (Ono et al. 1994), most of the homology is concentrated within the DEAD

Figure 6. UAP56 is recruited to the pre-mRNA in a U2AF⁶⁵-dependent manner. A schematic representation of the experimental protocol is shown *below* the autoradiograph. The indicated biotinylated RNAs were incubated with nuclear extract (lanes 1–3), or a biotinylated wild-type pre-mRNA was incubated with U2AF-depleted nuclear extract (lane 4) under standard conditions. Reactions were terminated by addition of heparin (200 ng/ml). After binding of complexes to avidin-agarose beads and extensive washing, the affinity-purified complexes were digested with RNase A, and released UAP56 detected by immunoblotting. (Δ5' pre-mRNA) Adenovirus major late promoter (AdMLP) pre-mRNA substrate lacking the first exon and 5' splice site; (ΔPY-tract pre-mRNA) AdMLP pre-mRNA substrate in which the Py tract was substituted by a T7 promoter sequence.

box helicase motifs. Thus, it remains to be determined whether UAP56 is in fact the human homolog of yeast PRP5.

U2AF⁶⁵ is also highly conserved; for example, human U2AF⁶⁵ is 84% and 53% homologous to its *Drosophila* (Kanaar et al. 1993) and *Schizosaccharomyces pombe* (Potashkin et al. 1993) counterparts, respectively. Interestingly, the U2AF⁶⁵ linker region is particularly well conserved; for example, positions 146 to 153 of U2AF⁶⁵, which are within the UAP56 binding site, contain seven out of eight identical amino acids (146-RQARRLYV-153) in all known U2AF⁶⁵ sequences from *S. pombe* to man.

Model for UAP56 function

Based on the results presented here, we suggest that UAP56 is recruited to the pre-mRNA through interac-

tion with U2AF⁶⁵ bound at the Py-tract. An attractive possibility is that UAP56 catalyzes an ATP-dependent conformational change in the pre-mRNA branchpoint or U2 snRNA that facilitates the entry of U2 snRNP into the spliceosome and possibly also affects subsequent steps. U2AF is destabilized following entry of U2 snRNP (Bennett et al. 1992), and it will be important to determine whether UAP56 is also associated with the spliceosome only transiently.

U2 snRNA undergoes several conformational changes during splicing. For example, the secondary structure of U2 snRNA is altered to form two base-pairing interactions with U6 snRNA (Datta and Weiner 1991; Wu and Manley 1991; Madhani and Guthrie 1992, 1994; Sun and Manley 1995). UAP56 could facilitate such rearrangements, perhaps only following establishment of U2 snRNA-branchpoint base-pairing, an interaction stabilized by the U2AF⁶⁵ RS domain (Valcárcel et al. 1996). According to this model, U2AF⁶⁵ would act as a cofactor for UAP56 activity. In addition, stem-loop IIa of U2 snRNA can form an alternative structure that renders U2 snRNP unable to interact with the branchpoint (Ares and Igel 1990; Zavaneli and Ares 1991). Thus, a possible function of UAP56 may be to unwind this alternative structure, thereby promoting formation of stem IIa. Finally, analogous to yeast PRP16 (Burgess and Guthrie 1993), UAP56 could be part of a proof reading mechanism to reverse or discard anomalous branchpoints.

The U2AF⁶⁵-UAP56 interaction may also be relevant to previous studies demonstrating that distance from the Py tract/3' splice site is a critical determinant of mam-

Figure 7. UAP56 is required for the U2 snRNP-branchpoint interaction. (A) Analysis of U2AF⁶⁵ in-frame deletion mutants. Complex A formation on the Δ5' AdMLP pre-mRNA substrate was analyzed by native gel electrophoresis under standard splicing conditions in nuclear extract (lane 1), U2AF⁶⁵-depleted nuclear extract (lane 2), or U2AF⁶⁵-depleted nuclear extract supplemented with the indicated U2AF⁶⁵ derivative to a final concentration of 25 ng/μl (lanes 3–6). (FL) Full-length U2AF⁶⁵. (B) Biochemical complementation assay. Complex A formation on the Δ5' AdMLP pre-mRNA substrate was analyzed by native gel electrophoresis under standard splicing conditions in nuclear extract (lane 7), mock-depleted nuclear extract (lane 8), UAP56-depleted nuclear extract (lane 9), or UAP56-depleted nuclear extract supplemented with immunopurified UAP56 to a final concentration of 10 ng/μl (lane 10).

malian branchpoint selection (for review, see Green 1986). Recruitment of a factor required for U2 snRNP assembly by Py tract-bound U2AF⁶⁵ would help to explain this distance constraint.

Conservation of components involved in the U2 snRNP-branchpoint interaction

Many of the human factors that mediate the U2 snRNP-branchpoint interaction have known yeast homologs, including U1 and U2 snRNPs, and at least five of seven subunits of the protein splicing factors SF3a and SF3b (Bennett and Reed 1993; Brosi et al. 1993; Legrain and Chapon 1993; Gozani et al. 1996; Wells et al. 1996). Our finding that humans, like yeast, contain a DEAD box protein required for U2 snRNP assembly emphasizes the conservation of components and mechanisms involved in the U2 snRNP-branchpoint interaction.

In yeast, DEAD box proteins are known to be involved at virtually every step of the splicing reaction (for review, see Madhani and Guthrie 1994; Kramer 1996). In the mammalian system, far fewer DEAD box proteins have been identified (Ono et al. 1994; Lauber et al. 1996; this report). The finding of a DEAD box protein involved in the U2 snRNP-branchpoint interaction suggests that significant mechanistic differences between the two systems are unlikely. In mammalian systems, biochemistry has been used primarily to identify splicing factors, whereas in yeast DEAD box proteins have been discovered through genetic approaches. Perhaps new mammalian DEAD-box splicing factors may best be identified through approaches like the two-hybrid screen used here.

In light of the strong conservation of the pre-mRNA splicing machinery, it remains surprising that yeast lack an obvious homolog of U2AF⁶⁵. Several candidates have been proposed (Birney et al. 1992; Abovich et al. 1994) but strong functional evidence for their role as a U2AF⁶⁵ homolog is lacking. We have shown that an additional function of U2AF⁶⁵ is recruitment of the DEAD box protein UAP56 to the branchpoint region. Therefore, determining the components that direct DEAD box proteins into the pre-spliceosome may shed light on the yeast factors that act similarly to U2AF⁶⁵.

Materials and methods

Construction and expression of U2AF⁶⁵ derivatives

Construction of GST-U[1-233]Sx has been described (Valcárcel et al. 1993). The GST-U[1-55]Sx construct was obtained from pGEX-3X-U[1-233]Sx by digestion with *Xma*I and *Stu*I, followed by Klenow treatment and religation of the plasmid. The GST-U[56-233]Sx construct was similarly obtained from pGEX-3X-U[1-233]Sx by deletion of the *Bam*HI-*Xma*I fragment, Klenow treatment, and religation of the plasmid. Internal deletions in U2AF⁶⁵ were created by PCR using appropriate primers. All proteins were expressed in *E. coli* as fusions with GST and purified as described previously (Lin and Green 1991).

Expression and purification of recombinant UAP56

Bacterially expressed UAP56 was produced in and purified from

E. coli as a GST fusion protein by standard procedures. For production in baculovirus, a complete UAP56 cDNA was cloned into baculovirus transfer vector pBlueBacHis2C (Invitrogen). His-tagged UAP56 was purified from infected insect cells on Ni²⁺-agarose according to the manufacturer's (Invitrogen) protocols, dialyzed against buffer D [20 mM HEPES at pH 8.0, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol (DTT), 0.1 M KCl], loaded on a DEAE column, and recovered in buffer D containing 0.3 M KCl.

Splicing and spliceosome assembly assays

Splicing assays, and the analysis of spliced products by primer extension using splice junction oligonucleotides, were performed as described (Valcárcel et al. 1993). Nuclear extracts were prepared as in Dignam et al. (1983), and U2AF-depleted nuclear extracts were prepared as in Zamore and Green (1991), except that chromatography was performed with oligo(dT)-cellulose instead of poly(U)-Sephadex.

Native gel assays for spliceosome assembly were performed as described in Zamore and Green (1991). For affinity purification of RNA-protein complexes, biotinylated RNAs were incubated with nuclear extract under conditions that allow spliceosome formation and reactions terminated with heparin (200 ng/μl). After binding of complexes to avidin-agarose beads and five 10-min washes in buffer R (20 mM Tris-HCl at pH 7.8, 0.1% Triton X-100, 150 mM KCl, 2.5 mM EDTA), the affinity-purified complexes were digested with RNase A to release proteins.

Yeast two-hybrid screen

Two-hybrid assays were performed as described by Gyuris et al. (1993). Briefly, full-length U2AF⁶⁵ fused to the LexA DNA-binding domain in the vector pEG202 was used as "bait," and a HeLa cDNA library fused to the procaryotic B42 acidic activation domain as "prey." In the library vector pJG4 expression of the prey fusions is driven by the galactose-inducible promoter GAL1, allowing for elimination of false positives by testing colonies on glucose and galactose medium (the plasmids pJG4, pEG202, and pSH18-34 and the EGY48 yeast strain were a gift from R. Brent [Harvard Medical School, Boston, MA]). Colonies harboring interacting partners were selected by their ability to grow on Leu⁻-galactose medium. Interaction between bait and prey was verified by galactose inducible blue-staining on X-gal-containing medium. Library plasmids containing positives were rescued into KC8 bacteria by selection on Trp⁻ medium.

cDNAs were analyzed first by restriction mapping using 4-bp cutters and sorted into classes based on their restriction map pattern. At least one representative cDNA from each class was sequenced partially by the dideoxy method using a commercially available kit (U.S. Biochemical). Subsequently, nonspecific interacting proteins were eliminated by screening positives against LexA alone (empty vector) or a LexA-Rex fusion protein. This eliminated 14 clones leaving 55 clones as U2AF⁶⁵-specific interacting proteins (see Table 1). Two independent plasmids were isolated from the screen, one (pJG6519) encoding UAP56 starting from amino acid 14, and a second (pJG6553) encoding UAP56 starting from amino acid 42. The insert of pJG6519 was subcloned and sequenced. The 5' end containing the amino-terminal amino acids was obtained by PCR and sequenced.

Immunodepletion of UAP56 and complementation assays

Antibodies were raised in rabbits (Babco) by immunization with a GST-UAP56 fusion protein. UAP56-depleted nuclear extracts

(ΔUAP56 NE) were prepared by incubating 150 μl of HeLa nuclear extract for 2 hr at 4°C with 50 μg of affinity-purified α-UAP56 antibody cross-linked to 25 μl of protein A beads. The beads were separated from the extract by centrifugation in a microcentrifuge. Two microliters of nuclear extract or UAP56-depleted nuclear extract were used per splicing reaction in a final volume of 10 μl. The reactions were carried out under standard condition at 30°C for 1 hr.

For immunopurification of UAP56, HeLa nuclear extract was dialyzed against buffer A (20 mM HEPES at pH 7.9, 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.1 M KCl, and 10% glycerol) and fractionated by chromatography on phosphocellulose. Five milliliters of the 0.1 M KCl fraction was incubated for 2 hr at 4°C with affinity-purified α-UAP56 antibody cross-linked to protein A-agarose beads. The immunoprecipitates were washed in buffer A (400 mM KCl), and UAP56 eluted with 100 mM glycine at pH 2.8 followed by neutralization with one-tenth vol. of 1 M Tris-HCl at pH 8.0.

Immunoprecipitation analysis

Ten milliliters of α-UAP56 antibody or preimmune serum were cross-linked to 3 ml of protein A beads (Repligen) using dimethylpylmalate (20 mM). Fifty microliters of washed beads were equilibrated in buffer D containing 0.1 M KCl and incubated with 25 μl HeLa nuclear extract for 2 hr at 4°C. Precipitates were washed three times in 500 μl of buffer D containing 0.1 M KCl and bound proteins eluted in 100 μl of buffer D containing 2.5M KCl. Eluates were electrophoresed on 10% SDS-PAGE and blotted onto a PVD membrane for immunoblot analysis. Coimmunoprecipitated U2AF⁶⁵ was detected using a monoclonal α-U2AF⁶⁵ antibody (a gift from M. Carmo-Fonseca, University of Lisbon, Portugal).

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