The splicing factor U2AF³⁵ mediates critical protein–protein interactions in constitutive and enhancer-dependent splicing

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The splicing factor U2AF (U2 snRNP auxiliary factor) is a heterodimer with subunits of 65 and 35 kD (U2AF⁶⁵ and U2AF³⁵). U2AF⁶⁵ binds specifically to 3' splice sites, but previous studies failed to demonstrate a function for U2AF³⁵. Here, we report that U2AF³⁵ is required for constitutive splicing and also functions as a mediator of enhancer-dependent splicing. Nuclear extracts deficient in U2AF³⁵ were inactive; however, both constitutive and enhancer-dependent splicing could be restored by the addition of purified recombinant U2AF³⁵. In vitro protein–RNA interaction studies with pre-mRNAs containing either a constitutive or regulated splicing enhancer revealed that U2AF³⁵ directly mediates interactions between U2AF⁶⁵ and proteins bound to the enhancers. Thus, U2AF³⁵ functions as a bridge between U2AF⁶⁵ and the enhancer complex to recruit U2AF⁶⁵ to the adjacent intron.

[**Key Words:** U2AF; RNA; splicing; pre-mRNAs; SR proteins]

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The production of metazoan messenger RNAs (mRNAs) requires the accurate removal of introns from pre-messenger RNAs (pre-mRNAs) by RNA splicing [for review, see Moore et al. 1993]. Initial recognition of the correct pairs of 5' and 3' splice sites by the splicing apparatus is a critical step in the processing of both constitutively and alternatively spliced pre-mRNAs [for review, see Rio 1992; Black 1995; Berget 1995; Reed 1996]. The splicing factor U2AF (U2 snRNP auxiliary factor; Ruskin et al. 1988) plays a central role in the initial recognition of 3' splice sites [Bennett et al. 1992; Michaud and Reed 1993; for review, see Fu 1995]. U2AF is a heterodimer consisting of 35- and 65-kD subunits (U2AF³⁵ and U2AF⁶⁵) [Zamore and Green 1989]. U2AF⁶⁵, which is an essential splicing factor, contains three RNA recognition motifs (RRMs) and binds specifically to the pyrimidine tract at the 3' splice site [Zamore et al. 1992]. At present, the function of U2AF³⁵ is not understood [Zamore and Green 1991], but a number of observations suggest that it might play an essential role in constitutive splicing. First, both subunits of U2AF are highly conserved between *Drosophila* and mammals, and the genes encoding the *Drosophila* U2AF³⁵ and U2AF⁶⁵ homologs are required for viability [Kanaar et al. 1993; D. Rio, pers. comm.]. Second, both U2AF⁶⁵ and U2AF³⁵ are present in the E complex, the earliest known mammalian spliceo-

somal complex [Bennett et al. 1992]. Third, protein–protein interaction studies suggest an important function for U2AF³⁵ in constitutive splicing [Wu and Maniatis 1993]. U2AF³⁵, but not U2AF⁶⁵, specifically interacts with members of the SR family of general splicing factors in the yeast two-hybrid system and in vitro [Wu and Maniatis 1993].

SR proteins [Zahler et al. 1992], which promote U1 snRNP and U2AF binding during the E complex assembly [Kohtz et al. 1994; Staknis and Reed 1994], contain an RRM and a serine–arginine (SR)-rich domain [for review, see Fu 1995]. The RRM is required for specific RNA binding, whereas the SR domain is necessary for protein–protein interactions [Wu and Maniatis 1993; Amrein et al. 1994; Kohtz et al. 1994]. SR proteins can interact simultaneously with the integral U1 snRNP protein U1-70K [Wu and Maniatis 1993; Kohtz et al. 1994] and with U2AF³⁵ [Wu and Maniatis 1993]. Similarly, U2AF³⁵ contains at least two protein–protein interaction domains: a region required for dimerization with U2AF⁶⁵ [Zhang et al. 1992], and an SR domain [J. Wu and T. Maniatis, unpubl.]. Thus, U2AF³⁵ may form a bridge between U2AF⁶⁵ bound to the 3' splice site and U1 snRNP bound to upstream or downstream 5' splice sites [Wu and Maniatis 1993]. In the absence of evidence for a function of U2AF³⁵ in constitutive splicing, however, the significance of these protein–protein interactions remains to be established.

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U2AF³⁵ may also be required for the function of exonic splicing enhancers, regulatory RNA sequences located in exons downstream from weak 5' or 3' splice sites (Wu and Maniatis 1993). Two types of splicing enhancers have been identified: One type functions constitutively (Lavigne et al. 1993; Sun et al. 1993; Watakabe et al. 1993; Xu et al. 1993; Huh and Hynes 1994; Tanaka et al. 1994), whereas the other is regulated (Ryner and Baker 1991; Tian and Maniatis 1992, 1993). Constitutive enhancers are recognized by specific SR proteins (Lavigne et al. 1993; Sun et al. 1993; Staknis and Reed 1994; Tacke and Manley 1995), which stimulate the formation of ribonucleoprotein enhancer complexes (Staknis and Reed 1994).

The *doublesex* repeat element [*dsx* repeat element (*dsxRE*)] is a regulated splicing enhancer that is required for female-specific splicing of *dsx* pre-mRNA. The *dsxRE* consists of six 13-nucleotide repeat sequences (R1–R6; Baker 1989) and a purine-rich element (PRE), which together activate a weak female-specific 3' splice site located 300 nucleotides upstream (Lynch and Maniatis 1995). The activity of the *dsxRE* requires the regulatory proteins Transformer (Tra) and Transformer 2 (Tra2) (Nagoshi and Baker 1990; Hedley and Maniatis 1991; Hoshijima et al. 1991; Ryner and Baker 1991) and one or more members of the SR family of general splicing factors (Tian and Maniatis 1993).

Tra, Tra2, and one or more members of the SR family of general splicing factors can form a stable complex on the *dsxRE* (Tian and Maniatis 1993, 1994), which is sufficient to commit *dsx* pre-mRNA to enhancer-dependent, female-specific splicing in vitro (Tian and Maniatis 1993). In vitro binding studies with purified recombinant proteins revealed that Tra, Tra2, and SR proteins bind cooperatively and specifically to the *dsxRE* (Lynch and Maniatis 1995). Both Tra and Tra2 contain SR domains, whereas Tra2, but not Tra, contains an RRM. Thus, the cooperative assembly of the *dsx* enhancer complex involves both protein–protein and protein–RNA interactions. The possibility that U2AF³⁵ may function to link the *dsx* enhancer complex to U2AF⁶⁵ at the female-specific splice site was suggested by the observation that Tra and Tra2 interact with each other and with U2AF³⁵ in the yeast two-hybrid system and in vitro (Wu and Maniatis 1993; Amrein et al. 1994).

Although enhancers have been shown to promote the binding of U2 snRNP to the branchpoint sequence (Lavigne et al. 1993) and U2AF to weak 3' splice sites (Hoffman and Grabowski 1992; Wang et al. 1995), there is no direct evidence that U2AF³⁵ is required. In addition, previous studies of the role of U2AF in constitutive splicing led to the conclusion that U2AF⁶⁵, but not U2AF³⁵, is an essential splicing factor (Zamore and Green 1991). In this paper we investigate the role of U2AF³⁵ in both constitutive and enhancer-dependent splicing. We found that U2AF³⁵ is an essential splicing factor that functions as a critical component in a network of RNA-binding proteins that link splicing enhancers with weak 3' splice sites.

Results

U2AF³⁵ is required for constitutive and enhancer-dependent pre-mRNA splicing

Previously, U2AF⁶⁵ was shown to be an essential splicing factor (Zamore and Green 1989, 1991). In these studies the U2AF heterodimer was depleted from HeLa cell nuclear extracts by virtue of the affinity of U2AF⁶⁵ for immobilized poly(U) RNA. These U2AF-depleted extracts were unable to splice pre-mRNA; however, splicing could be reconstituted by the addition of purified U2AF⁶⁵. In contrast, U2AF³⁵ was inactive in this assay (Zamore and Green 1991). Thus, U2AF³⁵ did not appear to be required for constitutive splicing. It is possible, however, that depletion of U2AF³⁵ was incomplete, and the remaining U2AF³⁵ complemented the added U2AF⁶⁵. To examine this possibility, we probed Western blots of poly(U)-depleted nuclear extracts with polyclonal antibodies raised separately against purified U2AF⁶⁵ and U2AF³⁵ expressed in baculovirus. As shown in Figure 1A, U2AF⁶⁵ was not detected in the poly(U)-depleted extracts, but a low level of U2AF³⁵ remained (lanes 4–6). Thus, there must be more U2AF³⁵ than U2AF⁶⁵ in nuclear extracts, or a small amount of U2AF³⁵ dissociates from the bound U2AF⁶⁵ during the depletion process.

As an alternative assay for U2AF function, we attempted to remove the heterodimer from nuclear extracts by use of the polyclonal antibodies described above. Although we were unable to accomplish this with the U2AF⁶⁵ antibodies, ~80% of both U2AF subunits could be removed with the U2AF³⁵ antiserum (Fig. 1A, cf. lanes 7–9 and 10–12). Mock depletion had no effect on the level of either U2AF subunit (cf. lanes 7–9 to lanes 1–3). To determine whether the partial depletion of both U2AF subunits resulted in a loss of splicing activity, we tested two constitutively spliced pre-mRNAs in the immunodepleted extracts. As expected, both human β -globin, and adenovirus major late (AdML) pre-mRNA, were spliced in the mock-depleted extract (Fig. 1B, lanes 1,6). In contrast, neither pre-mRNA was spliced in the nuclear extract immunodepleted with the anti-U2AF³⁵ antibody (lanes 2,7). Addition of either U2AF³⁵ (lanes 3,8) or U2AF⁶⁵ (lanes 4,9) to the immunodepleted extracts, however, partially restored the splicing of both RNA substrates, and splicing could be fully reconstituted by addition of both U2AF subunits (Figure 1B, lanes 5,10). We conclude that the low levels of U2AF³⁵ and U2AF⁶⁵ in the immunodepleted extracts were insufficient for splicing but were sufficient to partially restore splicing when the other subunit was added to the extract as recombinant protein. Splicing was completely restored when both subunits were added. Thus, both U2AF³⁵ and U2AF⁶⁵ are required for constitutive splicing. The splicing activity observed with U2AF was specific, because the addition of an excess of the individual recombinant SR proteins SC35, SF2/ASF, as well as Tra or Tra2, did not restore splicing activity to the immunodepleted nuclear extracts (data not shown).

To determine whether constitutive enhancer-depen-

Figure 1. U2AF³⁵ is required for both constitutive and enhancer-dependent splicing. (A) Detection of U2AF⁶⁵ and U2AF³⁵ in poly(U)-depleted and immunodepleted nuclear extracts. Three different amounts of nuclear extract (NE) or nuclear extracts that were poly(U)-depleted (Poly-U depl.), mock-depleted (Mock), or anti-U2AF³⁵-depleted (α U2AF³⁵ depl.) (containing 135, 45, and 15 μ g of total nuclear proteins, respectively) were fractionated on a 10% polyacrylamide SDS gel, transferred to nitrocellulose, and probed with a combination of U2AF³⁵ and U2AF⁶⁵ rabbit polyclonal antisera. Positions of the U2AF⁶⁵ and U2AF³⁵ bands are indicated at the left, and the smears between the two proteins are the IgG molecules from the rabbit sera. (B) U2AF³⁵ is required for constitutive splicing. Human β -globin and AdML splicing constructs are illustrated at the top. Splicing products are indicated at the right and left. Extracts and proteins used in each experiment are listed above the lanes: (Lanes 1,6) Mock extract; (lanes 2,7) extract immunodepleted with anti-U2AF³⁵ antiserum; (lanes 3–5, 8–10) immunodepleted extracts supplemented with baculovirus-expressed purified recombinant U2AF³⁵ alone (lanes 3,8), U2AF⁶⁵ alone (lanes 4,9), or the combination U2AF³⁵ and U2AF⁶⁵ (lanes 5,10). (–) No U2AF was present in the reaction. (C) U2AF³⁵ is required for constitutive enhancer activity. The splicing substrate is *dsxR2-5*. Positions of the bands corresponding to precursor and spliced product are indicated at the right. Splicing conditions are similar to those described in B. (D) U2AF³⁵ is required for Tra- and Tra2-dependent splicing enhancer activity. Splicing construct is *dsxR1-5PRE*. Extracts and proteins used in the splicing reactions are listed above the panel. (+) Presence of Tra and Tra2 proteins; (–) no Tra/Tra2 or U2AF proteins.

dent splicing requires U2AF³⁵, we tested the *dsxR2-5* pre-mRNA in the U2AF-immunodepleted extracts. This pre-mRNA contains the *dsx* female-specific intron and repeats 2–5 of the *dsxRE* inserted 100 nucleotides downstream from the female-specific 3' splice site. Previous studies showed that the *dsx* female-specific intron is not spliced in the absence of an enhancer (Tian and Maniatis 1992). When the *dsxRE* is located in its normal position, 300 nucleotides from the 3' splice site, Tra and Tra2 are required for splicing (Tian and Maniatis 1992). When only 100 nucleotides of RNA separate the 3' splice site and enhancer, however, splicing occurs in the absence of Tra and Tra2 (Tian and Maniatis 1994). Thus, the R2-5 sequence functions as a constitutive enhancer in the RNA splicing substrate illustrated in Figure 1C. As expected, this RNA was spliced in the mock-depleted extract (Fig. 1C, lane 1) but not in the U2AF-depleted extract (lane 2). Enhancer-dependent splicing could be partially reconstituted by the addition of purified recombinant U2AF³⁵ alone (lane 3) or U2AF⁶⁵ alone (lane 4), however, and the level of splicing observed with both U2AF subunits together was indistinguishable from that of the mock-depleted extract (lane 5). The same result was obtained when a purine-rich enhancer from the

avian sarcoma leukemia virus (ASLV; Fu et al. 1991; Tanaka et al. 1994) was inserted 100 nucleotides downstream from the female-specific 3' splice site (data not shown).

To determine whether U2AF³⁵ is also required for regulated enhancer-dependent splicing, we tested the RNA splicing substrate illustrated in Figure 1D (*dsxR1-5PRE*). In this RNA the *dsxRE* lacking only repeat 6 is located in its normal position, 300 nucleotides downstream from the female-specific 3' splice site. Consistent with previous studies (Tian and Maniatis 1992; Lynch and Maniatis 1995), this RNA was spliced only in the presence of Tra and Tra2 (Fig. 1D, cf. lanes 1 and 2). Splicing was not observed in the U2AF-depleted extracts in the absence or presence of Tra and Tra2 (Fig. 1D, lanes 3,4). Tra- and Tra2-dependent splicing, however, was partially reconstituted with U2AF³⁵ or U2AF⁶⁵ alone and was fully reconstituted by the addition of both proteins (Fig. 1D, lanes 5–7).

These data show that constitutive splicing and both types of enhancer-dependent splicing require U2AF³⁵. It is possible, however, that the U2AF³⁵ requirement for enhancer-dependent splicing is a secondary consequence of its requirement for constitutive splicing. To investi-

gate this possibility we have examined the mechanisms involved in enhancer-dependent splicing and the role of U2AF³⁵ in this process.

Enhancer-dependent E complex formation and U2AF binding to the weak female-specific 3' splice site

Previous studies have shown that exonic enhancers can stimulate the formation of the spliceosomal complex A, the earliest ATP-dependent complex (Lavigne et al. 1993; Wang et al. 1995). Both subunits of U2AF, however, first associate with pre-mRNA during the assembly of the E complex, the earliest functional complex formed in the absence of ATP (Michaud and Reed 1991). Therefore, we carried out experiments to determine whether splicing enhancers promote E complex formation on the *dsx* pre-mRNA bearing a splicing enhancer.

When pre-mRNAs containing a strong 3' splice site are incubated in nuclear extracts in the absence of ATP, a mixture of the nonspecific H complex and the functional E complex is formed, and these two complexes can be separated by gel filtration (Michaud and Reed 1991). The amount of E complex formed can be enhanced significantly by the addition of SR proteins to the nuclear extracts, indicating that SR proteins are limiting in these extracts (Staknis and Reed 1994). In contrast, the *dsx* pre-mRNA lacking the *dsxRE* formed only H complex under these conditions, and the addition of SR proteins had no effect (Fig. 2A, panels 1,2). Thus, the weak female-specific splice site is not recognized by the splicing machinery in the absence of an enhancer, and SR proteins are unable to drive complex formation.

To examine the effect of a constitutive *dsx* splicing enhancer activity on E complex formation, we tested the *dsxR2-5* RNA in which repeats 2–5 are located within 100 nucleotides of the 3' splice site. As shown in Figure 2A, E complex formation was observed with this RNA, and the addition of SR proteins led to a significant increase in the amount of complex formed (Fig. 2A, panels 3,4). Also, we observed enhancer-dependent E complex formation with the *dsx* pre-mRNA when the *dsxR2-5* was replaced by a purine-rich exonic enhancer from ASLV (Fu et al. 1991; Tanaka et al. 1994; ASLV data not shown).

To determine whether the enhancer-dependent E complexes are functional, we attempted to chase the RNA in these complexes into spliced product (Michaud and Reed 1991). As shown in Figure 2B, labeled RNA from the E complex formed on the substrate containing the *dsxR2-5* could be efficiently chased into spliced product in the presence of excess competitor RNA. In contrast, splicing was not observed with the RNA in the H complex fractions from the same gel-filtration column. These observations show that an exonic enhancer is required for the assembly of functional E complex on the weak *dsx* 3' splice site.

Both the 65- and 35-kD subunits of U2AF are present in E complexes formed on pre-mRNAs containing strong 3' splice sites (Zamore and Green 1991; Bennett et al. 1992). The pyrimidine tract of the *dsx* 3' splice site is

Figure 2. The *dsxRE* promotes the assembly of functional E complex on the *dsx* female-specific intron. (A) Detection of E complex assembly by gel filtration. RNA substrates used in the gel-filtration assay are illustrated at the top of each panel. Open boxes represent the *dsx* common exon 3; solid boxes represent the *dsx* female-specific exon; the broken line indicates the positions of the *dsxRE*. ³²P-labeled *dsxΔRE* or *dsxR2-5* was incubated in an ATP-depleted nuclear extract (panels 1,3) or in the same extract supplemented with ~2 μg of purified total SR proteins from HeLa cells (panels 2,4). The resulting RNA-protein complexes were fractionated on a gel-filtration column, and the radioactivity present in each fraction was determined in a scintillation counter (Reed 1990). Peaks corresponding to E and H complexes are indicated, and peaks to the left and right of these complexes correspond to the void volume and degraded pre-mRNA, respectively. (B) The E complex formed on the *dsxR2-5* RNA is a functional intermediate in spliceosome assembly. The fractions containing *dsxR2-5* RNA from either the E complex or the H complex were complemented with nuclear extracts in the presence of 0-, 40-, and 80-fold molar excess of cold *dsxR2-5* RNA. RNA prepared from the unchased E or H complexes is shown in lane 1 or lane 5.

interrupted by purines, however, and is therefore not a high-affinity binding site for U2AF⁶⁵. To determine whether splicing enhancers promote the binding of U2AF to this weak 3' splice site during E complex formation, we carried out Western blotting experiments with affinity-purified E complexes (Bennett et al. 1992). As expected from earlier studies (Michaud and Reed 1993), both U2AF⁶⁵ and U2AF³⁵ were present in the E complex formed on the strong AdML 3' splice site (Fig. 3). Only small amounts of U2AF were detected in H complex fractions derived from the same gel-filtration column, most likely caused by contaminating E complex. The *dsx* pre-mRNA lacking the *dsxRE* formed only the H complex (Fig. 2A), and this complex did not contain either subunit of U2AF (Fig. 3). In contrast, the E complex that formed on *dsx* RNAs bearing repeats 2–5 of the *dsxR2-5* contained both U2AF⁶⁵ and U2AF³⁵. Similarly, both subunits of U2AF were present in the E complexes formed on a *dsx* pre-mRNA bearing the ASLV splicing enhancer (*dsx3'* ASLV; Fig. 3). We conclude that constitutive splicing enhancers recruit U2AF³⁵ and U2AF⁶⁵ to E complexes formed on the female-specific *dsx* pre-mRNA.

Ribonucleoprotein complexes can be assembled on RNAs containing exonic enhancers but lacking the adjacent intron (Tian and Maniatis 1993; Staknis and Reed 1994). UV cross-linking experiments have shown that these complexes contain SR proteins but do not contain U2AF⁶⁵. As shown in Figure 3, enhancer complexes formed on the *dsxRE* (R2–5) and the ASLV enhancer (ASLV) contain significant amounts of U2AF³⁵. Roughly equal amounts of the two U2AF subunits are present in the E complexes of all of the RNAs containing a 3' splice site. In contrast, there is a preferential association of U2AF³⁵ with the RNAs containing an enhancer but lacking a 3' splice site (R2–5 and ASLV). A relatively small amount of U2AF⁶⁵ was associated with the R2–5 complex, whereas none was detected in the ASLV complex. Thus, the U2AF³⁵ subunit appears to associate preferentially with the enhancer complexes. This observation

suggests the interesting possibility that U2AF³⁵ may function as a component of enhancer complexes.

Tra- and Tra2-dependent E complex formation and U2AF binding to the weak female-specific 3' splice site

In the experiments described above the *dsxRE* was located 100 nucleotides downstream from the female-specific 3' splice site. Thus, splicing and E complex formation did not require Tra and Tra2 (Tian and Maniatis 1994). Therefore, we carried out experiments to determine whether the Tra- and Tra2-dependent splicing enhancer also recruits U2AF to the weak *dsx* 3' splice site. As shown in Figure 4A, the *dsx* pre-mRNA containing the *dsxRE* at its normal position is not assembled into the E complex in the absence of Tra and Tra2. Addition of excess SR proteins results in the appearance of only a minor shoulder on the H complex peak, probably because of the binding of SR proteins to the enhancer. In the presence of Tra and Tra2, however, a significant amount of E complex is observed, and the addition of SR proteins substantially increases the amount of E complex formed (Fig. 4A).

When Western blots of affinity-purified complexes were probed with U2AF antibodies, both U2AF³⁵ and U2AF⁶⁵ were detected in the *dsx* RNA complexes (Fig. 4B). This association was Tra- and Tra2-dependent and was stimulated by SR proteins. Thus, like the constitutive splicing enhancer activity, the *dsx*-regulated enhancer activity promotes E complex assembly and recruits both subunits of U2AF to the complex.

U2AF³⁵ is required for enhancer-dependent binding of U2AF⁶⁵ to the weak female-specific 3' splice site

To distinguish the role of U2AF³⁵ in constitutive splicing from its role in enhancer-dependent splicing, we carried out in vitro RNA-binding experiments using purified recombinant proteins. Specifically, we asked

Figure 3. U2AF⁶⁵ and U2AF³⁵ are present in affinity-purified nucleoprotein complexes. Nucleoprotein complexes were formed on biotinylated RNAs and then fractionated by gel filtration. The complex assembly reactions were scaled-up 5–20 times depending on the efficiency of E or enhancer complex (Enc) formation. The enhancer complex is formed on RNAs containing an enhancer but lacking a 3' splice site. Equimolar amounts of the complexes were then selected with avidin-agarose beads (see Materials and methods), and the eluted proteins fractionated on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with rabbit polyclonal anti-U2AF³⁵ and anti-U2AF⁶⁵ antibodies raised against baculovirus-expressed proteins. RNA constructs used in this analysis are illustrated.

Figure 4. Tra- and Tra2-dependent E complex formation. (A) Fractionation of Tra- and Tra2-dependent E complexes by gel filtration. Experiments were carried out as described in Fig. 2, except that the RNA substrate used was dsxR1-5PRE as indicated. dsxR1-5PRE and 30% ATP-depleted nuclear extract were incubated alone (1) or supplemented with total SR proteins (2), Tra and Tra2 proteins (3) or the combination of SR protein and Tra and Tra2 proteins (4). (B) Western blotting analysis of affinity-purified Tra- and Tra2-dependent complexes. Reaction volumes were scaled up to 8 ml. RNAs migrating at the position of the E complex were pooled and affinity purified. (+ and -) The presence or absence of Tra/Tra2 and/or SR proteins, respectively, as indicated at the left. (Left) Anti-U2AF⁶⁵ antibody; (right) anti-U2AF³⁵ antibody. U2AF proteins detected by the antibodies are indicated.

whether enhancer complexes assembled with purified components can promote U2AF⁶⁵ cross-linking to the female-specific 3' splice site and, if so, whether U2AF³⁵ is required. UV cross-linking studies were carried out by use of ³²P-labeled dsx pre-mRNAs with or without the dsxRE located within 100 nucleotides of the 3' splice site. Titration experiments were carried out to establish conditions in which each of the proteins was present in

limiting amounts in the binding assay (data not shown). Under these conditions, purified recombinant U2AF⁶⁵ alone did not cross-link in the presence or absence of the dsxR2-5 (Fig. 5A, lanes 1,9). Moreover, addition of limiting amounts of purified recombinant SR proteins did not stimulate cross-linking (Fig. 5A, lanes 3,5,7,11,13,15). The combination of limiting amounts of SR proteins and recombinant U2AF³⁵, however, resulted in U2AF⁶⁵ cross-linking in the presence, but not in the absence, of the dsxR2-5 (Fig. 5A, cf. lanes 6 and 8 with lanes 14 and 16). U2AF⁶⁵ cross-linking was observed in the presence of SRp40 (lane 6) and SC35 (lane 8) but not in the presence of SF2/ASF (lane 4). This observation is consistent with previous in vitro splicing studies, which showed that SRp40 or SC35, but not SF2/ASF, is capable of committing dsx pre-mRNA to the splicing pathway in the presence of Tra and Tra2 (Tian and Maniatis 1993). Thus, the requirements for U2AF⁶⁵ cross-linking correlate with the requirements for dsx enhancer-dependent in vitro splicing. In addition, SRp40, but not SF2/ASF, binds specifically to the dsxRE in vitro (Lynch and Maniatis 1995).

We note that in previous studies, SR proteins were shown to cross-link to the dsxRE in complexes formed in nuclear extracts (Tian and Maniatis 1993). Therefore, it was surprising that only U2AF⁶⁵ cross-linking was observed in the experiments of Figure 5. However, this is likely caused by the relative efficiency of cross-linking of U2AF⁶⁵ and SR proteins. The previous studies (Tian and Maniatis 1993) were carried out with the isolated dsxRE in the absence of an adjacent intron; therefore, the relative efficiencies of U2AF⁶⁵ and SR protein cross-linking would not have been noticed. In addition, SR proteins cross-link to naked RNA inefficiently relative to other proteins (R. Reed, pers. comm.).

UV cross-linking experiments were carried out with two additional constitutive enhancer constructs to test the generality of the conclusions reached above. First, the wild-type ASLV purine-rich enhancer was inserted 100 nucleotides downstream from dsx female-specific 3' splice site. In this case, U2AF³⁵-dependent cross-linking of U2AF⁶⁵ was observed in the presence of SRp40 (Fig. 5B, lane 6) but not in the presence of SF2/ASF or SC35 (Fig. 5A, lanes 4,8). This observation is consistent with previous studies, which showed that wild-type ASLV enhancer complexes contain SRp40 but not SF2/ASF or SC35 (Staknis and Reed 1994). Similarly, when a mutant of the ASLV enhancer was tested in the UV cross-linking assay, U2AF³⁵-dependent U2AF⁶⁵ binding was detected in the presence of SF2/ASF (lane 12) and SC35 (lane 16), but not SRp40 (lane 14). Previous studies showed that the mutant ASLV enhancer is functional in vitro and that it associates with SF2/ASF and SC35, but not SRp40 (Staknis and Reed 1994). Thus, there is a direct correlation between the proteins that associate with enhancer complexes in nuclear extracts and the proteins that promote U2AF⁶⁵ binding.

Finally, we carried out UV cross-linking experiments to determine whether U2AF³⁵ is required for regulated (Tra- and Tra2-dependent) recognition of the 3' splice

Figure 5. U2AF³⁵ is required for enhancer-dependent cross-linking of U2AF⁶⁵ to the female-specific 3' splice site. Effects of the other proteins on U2AF⁶⁵ cross-linking were examined at lower concentrations of U2AF⁶⁵. When all three proteins (U2AF⁶⁵, U2AF³⁵, and SR) are limiting, cross-linking of U2AF⁶⁵, but not of SR proteins, was observed. This occurs because the efficiency of cross-linking of SR proteins is less than that of U2AF⁶⁵. The cross-linked proteins were fractionated on a 10% polyacrylamide-SDS gel and visualized by autoradiography. The RNAs used for the binding studies are illustrated at the top (A–C). Different combinations of proteins present in each binding reaction are indicated above the binding results. (+) The presence of the specific protein listed at left. (A) U2AF³⁵ is required for constitutive enhancer-dependent U2AF⁶⁵ cross-linking. UV cross-linking was performed with the two RNA constructs *dsxR2-5* and *dsxΔRE*. (B) Distinct SR proteins are required for U2AF³⁵-dependent binding of U2AF⁶⁵ to RNAs with different enhancers. *dsx3'-ASLV* contains a wild-type purine-rich enhancer of ASLV, whereas *dsx3'-ASLV-6U* contains six substitutions of uridine for adenine (Staknis and Reed 1994). (C) Tra and Tra2 promote U2AF³⁵-dependent U2AF⁶⁵ binding. The RNA used here is *dsxR1-5PRE*.

site by U2AF⁶⁵. The *dsx* RNA splicing substrate containing the *dsxRE* located at its normal position, 300 nucleotides downstream from the female-specific 3' splice site, was used as a substrate in these experiments. As shown in Figure 5C (lane 6), cross-linking was observed only when Tra, Tra2, SC35, and both U2AF⁶⁵ and U2AF³⁵ were present in the binding reaction. SF2/ASF was unable to promote U2AF⁶⁵ cross-linking (lane 9), consistent with our previous observation that SC35, but not SF2/ASF, can promote Tra- and Tra2-dependent commitment complex of the *dsx* pre-mRNA (Tian and Maniatis 1993). We conclude that U2AF³⁵ is required for both constitutive and regulated enhancer-dependent U2AF⁶⁵ binding to the weak 3' splice site, and the type of SR protein required for cross-linking corresponds to the type required for enhancer-dependent splicing.

Protein–protein interaction domains of U2AF³⁵ are required for enhancer-dependent splicing

To test the possibility that U2AF³⁵ functions as a bridge

between U2AF⁶⁵ and SR proteins during spliceosome assembly (Wu and Maniatis 1993), we carried out experiments to determine whether deletion of the protein–protein interaction domains of U2AF³⁵ and U2AF⁶⁵ blocks their ability to reconstitute immunodepleted extracts. Amino acids 64–182 of U2AF⁶⁵ are required for its interaction with U2AF³⁵ (Zhang et al. 1992), whereas the SR domain of U2AF³⁵ is required for interactions with Tra, Tra2, and SR proteins (J. Wu and T. Maniatis, unpubl.). The wild-type and mutant proteins were expressed in bacteria, and the purified proteins assayed in the immunodepleted extracts. Significantly, in the presence of wild-type U2AF⁶⁵, U2AF³⁵ΔSR failed to restore enhancer-dependent splicing in the immunodepleted extract (Fig. 6A, lane 6), suggesting that the interactions mediated through SR domains are essential for enhancer-dependent splicing. Similarly, U2AF⁶⁵Δ35 (deletion of amino acids 95–138), which does not form a heterodimer with U2AF³⁵, cannot complement the splicing activity of U2AF³⁵ in immunodepleted extracts (Fig. 6A, lane 7).

The failure of the U2AF⁶⁵Δ35 protein to promote enhancer-dependent splicing could be the result of its inability to interact with U2AF³⁵. Alternatively, deletion of the U2AF³⁵ interaction domain may cause a structural alteration of U2AF⁶⁵, rendering the protein nonfunctional. To examine this possibility, we tested the activity of the deletion mutant in two assays. First, we found that deletion in U2AF⁶⁵Δ35 had no effect on the ability of the protein to specifically cross-link to the pyrimidine tract of the AdML 3' splice site (data not shown). Thus, the RNA-binding domain of the mutant protein is functional. Second, we compared the constitutive splicing activities of wild-type U2AF⁶⁵ and U2AF⁶⁵Δ35 in nuclear extracts in which U2AF³⁵ was immunodepleted. As expected, in the presence of U2AF³⁵ wild-type U2AF⁶⁵, but not U2AF⁶⁵Δ35, could restore the splicing of AdML construct (Fig. 6B, lanes 4,5). We found, however, that when the amount of both proteins was increased (3, 9, and 27 ng of protein) without added U2AF³⁵, U2AF⁶⁵ alone could fully activate splicing, whereas the same amount of the mutant protein only partially restored splicing activity (Fig. 6B, lanes 6–11). This observation is consistent with the possibility that wild-type U2AF⁶⁵ can effectively recruit the low level of U2AF³⁵ present in the immunodepleted extract, whereas the mutant protein can function in the absence of U2AF³⁵ at high concentrations. In any case, the fact that the mutant protein could restore splicing activity shows that the protein was not inactivated by the deletion.

We conclude that the specific protein–protein interaction domains of U2AF³⁵ and U2AF⁶⁵ are required for

enhancer-dependent splicing and for efficient constitutive splicing.

Discussion

The results presented here show that U2AF³⁵ is an essential splicing factor that is also required for the function of both constitutive and regulated splicing enhancers. The role of U2AF³⁵ in all of these processes is to mediate interactions between U2AF⁶⁵, the RNA binding subunit of U2AF, and splicing factors containing one or more SR domains. This function is made possible by the presence of two protein–protein interaction domains in U2AF³⁵: An amino-terminal SR-rich domain that is required for interactions with SR proteins and the splicing regulators Tra and Tra2 (J. Wu and T. Maniatis, unpubl.), and a carboxy-terminal domain required for the formation of the U2AF³⁵/U2AF⁶⁵ heterodimer (Zamore et al. 1992; Zhang et al. 1992).

A number of previous observations suggested that U2AF³⁵ might be an essential splicing factor, but direct evidence was lacking (for discussion, see Zamore and Green 1991). Here we show that both U2AF³⁵ and U2AF⁶⁵ can stimulate constitutive and enhancer-dependent splicing in U2AF-deficient nuclear extracts. In addition, we show that the two U2AF subunits can complement each other to fully reconstitute both types of splicing activities in these extracts. Finally, we show that efficient complementation requires the U2AF interaction domains of both proteins. These observations strongly support the conclusion that the intact U2AF

Figure 6. The protein–protein interaction domains of U2AF³⁵ and U2AF⁶⁵ are required for enhancer-dependent splicing and efficient constitutive splicing. Wild-type U2AF⁶⁵, U2AF³⁵, and the deletion mutants were expressed in bacteria and added to a U2AF-immunodepleted nuclear extract. 35ΔSR indicates U2AF³⁵ΔSR in which the SR domain of U2AF³⁵ is deleted, and 65Δ35 indicates U2AF⁶⁵Δ35 that lacks the U2AF³⁵ interaction domain. The splicing substrates used in the experiments are dsxR2-5 (A) and AdML. (B) Positions of the bands corresponding to the precursor and spliced product are indicated at the right of the autoradiographs.

heterodimer is required for both constitutive and enhancer-dependent splicing.

Two approaches were taken to address the role of U2AF³⁵ enhancer-dependent splicing. First, we showed that E complex formation on the weak *dsx* female-specific 3' splice site requires a splicing enhancer, and the resulting enhancer-dependent E complexes contain both subunits of U2AF. Thus, splicing enhancers recruit U2AF to the weak 3' splice site at the earliest detectable step of splice site recognition. Interestingly, we found that enhancer complexes formed on RNAs containing only an enhancer element without the adjacent intron contain detectable amounts of U2AF³⁵, but little if any U2AF⁶⁵. Thus, it is possible that free U2AF³⁵ might be recruited to enhancer complexes, which could, in turn, recruit U2AF⁶⁵ to the adjacent 3' splice site.

Second, we showed that purified recombinant Tra, Tra2, and SR proteins can promote enhancer-dependent cross-linking of U2AF⁶⁵ to the weak 3' splice site, and, most importantly, that U2AF³⁵ is required for this specific protein-RNA interaction. The significance of this result was enhanced by showing that the SR proteins required for U2AF⁶⁵ cross-linking are identical to those required for *in vitro* splicing. Thus, the simultaneous interaction between U2AF³⁵ and both U2AF⁶⁵ and SR proteins is required for splicing. Taken together, these observations strongly support the conclusion that U2AF³⁵ mediates interactions between enhancer complexes and regulated 3' splice sites.

It is important to note that in all of these experiments each of the components must be present in limiting amounts to observe specific protein requirements. For example, in the cross-linking experiments U2AF³⁵-independent cross-linking of U2AF⁶⁵ could be observed if much higher levels of U2AF⁶⁵ were added to the binding reaction. Similarly, the U2AF⁶⁵Δ35 mutant protein was capable of complementing a U2AF³⁵-depleted extract, but only at concentrations significantly greater than that of the wild-type U2AF⁶⁵. These observations are consistent with the idea that multiple weak interactions are required for 3' splice site recognition. Thus, when all of the components are limiting, the loss of one interaction is sufficient to inactivate the complex. Addition of a large excess of a particular component, however, may reconstitute splicing activity by compensating for the loss of the weak interaction.

The function of U2AF³⁵ in splice site recognition and enhancer-dependent splicing

Although the weakly conserved recognition sequences within the intron are sufficient for splicing in isolation, they are not sufficient for correct splice site recognition in natural pre-mRNAs containing multiple introns (for review, see Berget 1995; Black 1995; and Reed 1996). In these cases, recognition of the correct 3' splice appears to be accomplished through the formation of a network of protein-protein interactions extending across the exon to the downstream 3' splice site (Fig. 7A; Hoffman and Grabowski 1992; for discussion, see Reed 1996). A key

Figure 7. The role of U2AF³⁵ in constitutive and enhancer-dependent splicing. (A) A model for the role of U2AF³⁵ in constitutive splicing. As explained in the text, U2AF³⁵ could function as a bridge between U2AF⁶⁵ at the 3' splice site and U1 snRNP at the downstream (cross exon) or upstream (cross intron) 5' splice site. (B) A model for the role of U2AF³⁵ in constitutive enhancer-dependent splicing. When the *dsx*RE is located within 100 nucleotides of the 3' splice site, it can activate splicing in the absence of Tra and Tra2. This is a consequence of specific, but weak, interactions between SR proteins and the *dsx*RE, which leads to cooperative binding of SR proteins that interact weakly with sequences within the exon. With the short exon, these weak interactions are sufficient to establish a network of protein-protein interactions across the exon, which leads to the binding of U2AF⁶⁵ to the weak 3' splice site. U2AF³⁵ functions as a bridge between the SR proteins and U2AF⁶⁵. (C) A model for the role of U2AF³⁵ in regulated enhancer-dependent splicing. In this case, the *dsx*RE is located at its normal position, 300 nucleotides downstream from the 3' splice site. Here, the SR proteins bound to the *dsx*RE are unable to form a network of proteins across the exon because of its size and the random distribution of weak SR-binding sites across the exon. Thus, none of the proteins binds with sufficient stability to form a complex, and splicing does not occur. In the presence of Tra and Tra2, however, a highly stable complex including the SR proteins forms on the enhancer. This complex is then able to interact directly with U2AF³⁵ and thus promote the binding of U2AF⁶⁵ to the 3' splice site.

element in this model is the ability of U2AF³⁵ to form a bridge between U2AF⁶⁵ and SR proteins bound to the exon (Reed 1996). Similarly, U2AF³⁵ may play a crucial role in the interaction between the 5' and 3' splice sites by functioning as a bridge in the U1 70K–SR protein–U2AF network of protein interactions across the intron (Fig. 7A, cross-intron; Wu and Maniatis 1993).

Another important aspect of the cross-exon model is the effect of the number of nucleotides between the downstream 5' splice site and the upstream 3' splice site on splice site recognition (Berget 1995; Reed 1996). Metazoan internal exons are rarely >300 nucleotides and average between 150 and 200 nucleotides (Berget 1995). In addition, exons are required for splice site selection (for review, see Black 1995; Reed 1996), and SR proteins preferentially interact with exon sequences in constitutively spliced RNAs (Blencowe et al. 1994; O. Gozani and R. Reed, pers. comm.). Thus, the constraint on exon size may be a consequence of the requirement for formation of a bridge across the exon with SR proteins. If the exon is too long, the probability of the formation of a continuous protein network is decreased (Reed 1996).

The same rationale may explain the distance effects on the constitutive and regulated activities of the *dsxRE*. SR proteins are capable of binding to the *dsxRE* in the absence of Tra and Tra2; however, this interaction is not very specific and the affinity is low (Tian and Maniatis 1994; Lynch and Maniatis 1995). This complex, however, appears sufficiently stable to recruit U2AF to the 3' splice site when the enhancer is in close proximity. When the exon is short, SR proteins bound to the exon may interact with U2AF³⁵ on one side and the enhancer complex on the other to form a stable complex (Fig. 7B). When the enhancer is located at a distance, however, the weak interactions between SR proteins and the exon might not be sufficient to link the enhancer with the 3' splice site. In contrast, in the presence of Tra and Tra2, the complex formed on the enhancer is sufficiently stable to interact directly with U2AF at the 3' splice site (Fig. 7C). This model is supported by the observation that complexes containing a 3' splice site and enhancer are sufficiently stable to engage in *trans*-splicing with separate RNAs containing a 5' splice site (Bruzik and Maniatis 1995; Chiara and Reed 1995).

In summary, the data presented here, in conjunction with the results from previous studies, strongly support the conclusion that U2AF³⁵ plays a critical role in both constitutive and enhancer-dependent splicing by mediating essential protein–protein and protein–RNA interactions required for accurate 3' splice site selection.

Materials and methods

Proteins and antibodies

The cDNAs containing the complete coding regions of U2AF⁶⁵ (Zamore et al. 1992), U2AF³⁵ (Zhang et al. 1992), and SRp40 (Screaton et al. 1995) were cloned into the baculovirus expression vector pVL1392 (PharMingen). A synthetic oligonucleotide encoding the start codon and six histidine residues was inserted immediately upstream from the amino-terminal codon of the

cDNAs prior to baculovirus cloning. The recombinant proteins were expressed according to the protocols provided by the manufacturer (PharMingen) and purified as described previously (Tian and Maniatis 1993). For the purification, cell pellets were resuspended in lysis buffer containing 20 mM Tris HCl (pH 7.5), 150 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% NP-40, 1 mM DTT, and a cocktail of protein inhibitors, and rotated at 4°C for 30 min. The lysates were centrifuged for 30 min at 8000 rpm (HB-4 rotor), and the pellets resuspended in 4 ml of extraction buffer [6 M guanidine-HCl, 0.1 M NaH₂PO₄, 0.01 M Tris (pH adjusted to 8.0 with NaOH), 1% β-mercaptoethanol]. The resuspended pellets were sonicated for 2 × 20 sec, rotated at room temperature for 1 hr, and centrifuged in an SW55 rotor for 1 hr at 50,000 rpm. The supernatants were then loaded on a nickel column, and the recombinant proteins recovered by use of standard protocols (Qiagen). U2AF⁶⁵ and U2AF³⁵ were eluted at pH 6.3 and dialyzed against high salt BC850 [20 mM Tris-HCl at pH 8.0, 850 mM KCl, 20% glycerol]. In the case of SRp40, the protein was dialyzed against the same buffer except that the salt concentration was 100 mM KCl. The dialyzed SRp40 was purified further by MgCl₂ precipitation (Zahler et al. 1992). Baculovirus-expressed SC35, SF2/ASF, Tra, and Tra2 recombinant proteins were purified as described previously (Tian and Maniatis 1993), and the total HeLa cell SR proteins were purified as described previously (Zahler et al. 1992).

Bacterially expressed U2AF⁶⁵, U2AF³⁵, and their mutants U2AF³⁵ΔSR and U2AF⁶⁵Δ35 were produced by use of standard protocols. Individual cDNA clones were inserted into pRSET plasmids (Invitrogen), which contained coding sequences for His tags, and then introduced into *Escherichia coli*. The resulting His-tagged proteins were purified by the same procedure used to obtain the baculovirus-expressed U2AF³⁵ and U2AF⁶⁵. U2AF³⁵ΔSR was made by a carboxy-terminal deletion of wild-type U2AF³⁵, and the cDNA was cut by *Sma*I restriction enzyme, then filled in, and religated to the expression vector. U2AF⁶⁵Δ35 was made by PCR deletion of amino acids 95 and 138.

The anti-U2AF³⁵ and anti-U2AF⁶⁵ antibodies are rabbit polyclonal antibodies generated against purified baculovirus-expressed U2AF³⁵ and U2AF⁶⁵. For Western analysis, antibodies were diluted 3000 times before the incubation.

Splicing substrates and *in vitro* splicing reactions

The *dsx*ΔRE contains 84 nucleotides of *dsx* exon 3, the complete intron 3, and the first 70 nucleotides of the fourth exon (Tian and Maniatis 1994) followed by the polylinker sequence of pSP73 (Promega). When the plasmid was linearized with *Nde*I, the *dsx*ΔRE transcript was the same size as that of *dsx*R2-5. The *dsx*R2-5 construct contains the same *dsx* sequence as *dsx*ΔRE, and in addition it has a *dsxRE* fragment beginning at *Mlu*I and ending at a PCR-generated *Bam*HI site between the fifth repeat and the PRE (Lynch and Maniatis 1995). *dsx*R1-5PRE was generated from plasmid D1 (Tian and Maniatis 1992), which contains the entire wild-type *dsx* fourth exon. After linearization with *Dra*I, this construct was used as the template for the *dsx*R1-5PRE transcript. *dsx*3'-ASLV and *dsx*3'-ASLV6U contain 62 nucleotides (*Bst*XI site) of the intron 3 and the first 70 nucleotides of the fourth exon followed by ASLV or ASLV6U enhancer sequences (Staknis and Reed 1994). pAdML3', which contains the 3' portion of the AdML intron and exon 2, was a gift from R. Reed (Michaud and Reed 1993). The adenovirus construct pSPAd (Solnick 1985) was linearized with *Hinc*II. Human β-globin pre-mRNA was synthesized from pT7-Hβ, which was generated by ligation of the 497-bp *Hind*III–*Bam*HI human β-globin fragment into pSP73 according to Reed et al. (1988).

RNAs were transcribed with T7 polymerase and capped during transcription. RNAs for gel filtration and splicing were labeled with [³²P]UTP.

In vitro splicing reactions were carried out as described in Tian and Maniatis (1992). In chase experiments, fractions containing ~1 ng of *dsxR2-5* RNA from either the E complex or the H complex were complemented with 8 μ l of nuclear extract (in 25- μ l reactions) in the presence of excess amounts of cold competitor RNA *dsxR2-5* (0, 40, and 80 ng). The reactions were then incubated in the presence of ATP, MgCl₂, for 90 min at 30°C. For functional assays with immunodepleted extracts, titration experiments were carried out to determine the amount of nuclear extract required to observe efficient splicing with the mock-depleted extract and no splicing with the immunodepleted extract. Depending on the splicing constructs, ~3–7 μ l of nuclear extract was used for functional assay in 25- μ l splicing reactions.

Gel filtration and affinity purification

Gel-filtration and affinity-purification methods were carried out as described previously (Bennett et al. 1992). Nuclear extract was preincubated at room temperature for 30 min to deplete endogenous ATP, and E complexes (200 μ l reaction) were formed in the absence of ATP, MgCl₂, and creatine phosphate. The E complex reactions were incubated at 30°C for 40 min in 30% of NE, 100 mM of KCl, and 30–200 ng of ³²P-labeled RNA (as specified in the figure legends). The reactions were then loaded directly onto a Sephacryl S-500 column (Pharmacia) (1.5 $\times$ 50 cm). The column was equilibrated and run with 20 mM Tris-HCl (pH 7.5), 60 mM KCl, 2.5 mM EDTA, and 0.1% Triton X-100 at a flow rate of 8 ml/hr. Fractions (1.0 ml) were collected, and 0.2-ml samples of each fraction were quantitated by Cerenkov counting.

For affinity purification of the E complex, biotinylated pre-mRNAs (Grabowski and Sharp 1986) were synthesized with T7 polymerase in a standard transcription reaction that included 30 μ M biotinylated UTP (Enzo Biochemicals), and E complex assembly reactions were scaled-up 10–50 times. The peak fractions were pooled, and the salt concentration was adjusted with NaCl to 250 mM. Avidin-agarose (10 μ l/ml) (Vector Labs) was added to the pooled fraction and mixed overnight at 4°C. The avidin beads were then washed four times with the same buffer; RNase A (2 μ l of 2 mg/ml) was then added to the avidin-agarose and incubated at 37°C for 30 min. Proteins were eluted from the avidin beads by heating at 65°C (2 $\times$ 5 min) in a buffer consisting of 20 mM Tris (pH 7.6), 20 mM DTT, and 2% SDS. The proteins were precipitated with four volumes of acetone and resuspended in SDS sample buffer. The affinity-purified complexes were fractionated on a 10% SDS-polyacrylamide gel and analyzed by Western blotting according to standard enhanced chemiluminescence (ECL) procedures (Amersham).

RNA-binding assay

RNAs for UV cross-linking were uniformly labeled with [³²P]UTP, [³²P]ATP, [³²P]GTP, [³²P]CTP, with a 1:18 molar ratio of [³²P]NTP to cold NTP. RNAs were incubated in a boiling water bath for 3 min before being added to the binding reactions. RNA-binding conditions were essentially the same as those described in Zamore et al. (1992) and Zuo and Manley (1994): 20 mM Tris (pH 7.9), 0.025% NP-40, 150 mM KCl, 10% glycerol, 0.6 mg/ml of yeast tRNA, 0.02 mg/ml of BSA, and 50–100 fmoles of labeled RNA. Titration experiments were performed to optimize conditions for the cooperative binding assays. Thus, the amount of each protein was adjusted to the linear range of its

binding curve. For example, U2AF⁶⁵ alone does not bind to the weak 3' splice site at low concentrations, but does at 10-fold higher concentrations. By use of this information, it was possible to achieve conditions in which every protein was limiting in the binding reactions. The amounts of each protein used in the binding reactions were 40–100 ng of U2AF⁶⁵, 20–40 ng of U2AF³⁵, 25–50 ng of SRp40, 20–40 ng of SF2/ASF, 20–40 ng of SC35, 50 ng of Tra, and 60 ng of Tra2. It is important to note, however, that the percentage of active protein and the presence of inhibitory activities for each purified recombinant protein preparation are unknown. The amount of U2AF⁶⁵ required in the cross-linking experiments was established empirically. The purified recombinant proteins were incubated with RNA in binding buffer for 30 min at 30°C, and the RNA-protein complexes were irradiated at the distance of 6 cm with a handheld mineral light (UVP, Inc., model UVG-11; 254 nm) for 10 min. RNase A (2 μ g) was added, and the samples were incubated for 30 min at 37°C. An equal volume of 2 $\times$ SDS gel loading buffer was added to each sample, and aliquots were analyzed by 10% SDS-PAGE.

The RNA binding of U2AF⁶⁵ Δ 35 was also performed by UV cross-linking under the same conditions as described above. Bacterially expressed wild-type U2AF⁶⁵ and mutant U2AF⁶⁵ Δ 35 were cross-linked to uniformly labeled Ad3' construct (see Fig. 6B).

Immunodepletion and poly(U) depletion

Protein A (0.5 ml) immobilized on Trisacryl (Pierce) and suspended (1:1), was loaded onto a minicolumn and washed with 3 ml of IP wash buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, and 1 mg/ml of BSA). Anti-U2AF³⁵ polyclonal antibody or the pre-immune serum (0.5 ml) was diluted with 2 ml of IP wash buffer, and loaded onto a protein-A Trisacryl column five times. The columns were washed by 5 ml of wash buffer and 5 ml of buffer D (20 mM Tris at pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT). Nuclear extract (0.5 ml) was prewarmed at room temperature for 30 min and passed through an anti-U2AF³⁵ affinity column or a mock column made with pre-immune serum. After being passed through the columns five times at room temperature, extracts were either used immediately for splicing reactions or frozen until needed. Depletion of U2AF was monitored by Western blotting with anti-U2AF³⁵ and anti-U2AF⁶⁵ antibodies. Poly(U) depletion of U2AF from HeLa cell nuclear extract was carried out as described previously (Zamore and Green 1991).

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