The splicing factor U2AF^{35} 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 (U2AF^{65} and U2AF^{35}). U2AF^{65} binds specifically to 3' splice sites, but previous studies failed to demonstrate a function for U2AF^{35}. Here, we report that U2AF^{35} is required for constitutive splicing and also functions as a mediator of enhancer-dependent splicing. Nuclear extracts deficient in U2AF^{35} were inactive; however, both constitutive and enhancer-dependent splicing could be restored by the addition of purified recombinant U2AF^{35}. In vitro protein–RNA interaction studies with pre-mRNAs containing either a constitutive or regulated splicing enhancer revealed that U2AF^{35} directly mediates interactions between U2AF^{65} and proteins bound to the enhancers. Thus, U2AF^{35} functions as a bridge between U2AF^{65} and the enhancer complex to recruit U2AF^{65} 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 (U2AF^{35} and U2AF^{65}) (Zamore and Green 1989). U2AF^{65}, 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 and Green 1989). U2AF^{35}, 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 and Green 1991). At present, the function of U2AF^{35} is not understood (Zamore and Green 1991), although 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 U2AF^{35} and U2AF^{65} homologs are required for viability (Kanaar et al. 1993; D. Rio, pers. comm.). Second, both U2AF^{65} and U2AF^{35} are present in the E complex, the earliest known mammalian spliceosomal complex (Bennett et al. 1992). Third, protein–protein interaction studies suggest an important function for U2AF^{35} in constitutive splicing (Wu and Maniatis 1993). U2AF^{35}, but not U2AF^{65}, 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 U2AF^{35} (Wu and Maniatis 1993). Similarly, U2AF^{35} contains at least two protein–protein interaction domains: a region required for dimerization with U2AF^{65} (Zhang et al. 1992), and an SR domain (J. Wu and T. Maniatis, unpubl.). Thus, U2AF^{35} may form a bridge between U2AF^{65} 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 U2AF^{35} in constitutive splicing, however, the significance of these protein–protein interactions remains to be established.

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U2AF^{35} 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 and Maniatis 1993). Two types of splicing enhancers for female-specific splicing of pre-mRNA. The dsx factors (Tian and Maniatis 1993).

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 U2AF^{35} may function to link the dsx enhancer complex to U2AF^{65} at the female-specific splice site was suggested by the observation that Tra and Tra2 interact with each other and with U2AF^{35} 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 [Lavigneuer et al. 1993] and U2AF to weak 3' splice sites [Hoffman and Grabowski 1992; Wang et al. 1995], there is no direct evidence that U2AF^{35} is required. In addition, previous studies of the role of U2AF in constitutive splicing led to the conclusion that U2AF^{65}, but not U2AF^{35}, is an essential splicing factor [Zamore and Green 1991]. In this paper we investigate the role of U2AF^{35} in both constitutive and enhancer-dependent splicing. We found that U2AF^{35} 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
U2AF^{35} is required for constitutive and enhancer-dependent pre-mRNA splicing

Previously, U2AF^{65} 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 U2AF^{65} 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 U2AF^{65}. In contrast, U2AF^{35} was inactive in this assay [Zamore and Green 1991]. Thus, U2AF^{35} did not appear to be required for constitutive splicing. It is possible, however, that depletion of U2AF^{35} was incomplete, and the remaining U2AF^{35} complemented the added U2AF^{65}. To examine this possibility, we probed Western blots of poly(U)-depleted nuclear extracts with polyclonal antibodies that bind specifically to U2AF^{65} and U2AF^{35} expressed in baculovirus. As shown in Figure 1A, U2AF^{65} was not detected in the poly(U)-depleted extracts, but a low level of U2AF^{35} remained [lanes 4–6]. Thus, there must be more U2AF^{35} than U2AF^{65} in nuclear extracts, or a small amount of U2AF^{35} dissociates from the bound U2AF^{65} 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 U2AF^{65} antibodies, ~80% of both U2AF subunits could be removed with the U2AF^{65} antisera (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-U2AF^{35} antibody [lanes 2,7]. Addition of either U2AF^{35} [lanes 3,8] or U2AF^{65} [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 U2AF^{35} and U2AF^{65} 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 U2AF^{35} and U2AF^{65} 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 U2AF35 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 (Mock), mock-immunodepleted (Mock), or anti-U2AF35-depleted (αU2AF35 depl.) (containing 135, 45, and 15 μg 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 U2AF35 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), U2AF35 alone (lanes 4,9), or the combination U2AF35 and U2AF35 (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.

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 U2AF35 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 U2AF 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 (Lavigueur 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](link-to-figure) 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 position of splicing enhancers. (B) The E complex formed on the dsxR2-5 RNA is a functional intermediate in splicing 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 U2AF<sup>65</sup>. 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 U2AF<sup>65</sup> and U2AF<sup>35</sup> 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 complexes formed on a pre-mRNA bearing the ASLV enhancer (Fig. 3). As shown in Figure 4A, the dsx<sup>R2-5</sup> contained both U2AF<sup>65</sup> and U2AF<sup>35</sup>. Similarly, both subunits of U2AF were present in the E complexes formed on a dsx<sup>pre-mRNA</sup> bearing the ASLV splicing enhancer (dsx<sup>3′</sup> ASLV; Fig. 3). We conclude that constitutive splicing enhancers recruit U2AF<sup>35</sup> and U2AF<sup>65</sup> to E complexes formed on the female-specific dsx<sup>pre-mRNA</sup>.

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 U2AF<sup>65</sup>. As shown in Figure 3, enhancer complexes formed on the dsx<sup>RE</sup> [R2-5] and the ASLV enhancer [ASLV] contain significant amounts of U2AF<sup>35</sup>. 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 U2AF<sup>35</sup> with the RNAs containing an enhancer but lacking a 3′ splice site [R2-5 and ASLV]. A relatively small amount of U2AF<sup>65</sup> was associated with the R2-5 complex, whereas none was detected in the ASLV complex. Thus, the U2AF<sup>35</sup> subunit appears to associate preferentially with the enhancer complexes. This observation suggests the interesting possibility that U2AF<sup>35</sup> may function as a component of enhancer complexes.

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 U2AF<sup>35</sup> and U2AF<sup>65</sup> 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.

U2AF<sup>35</sup> is required for enhancer-dependent binding of U2AF<sup>65</sup> to the weak female-specific 3′ splice site

To distinguish the role of U2AF<sup>35</sup> 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. U2AF<sup>65</sup> and U2AF<sup>35</sup> 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-U2AF<sup>35</sup> and anti-U2AF<sup>65</sup> antibodies raised against baculovirus-expressed proteins. RNA constructs used in this analysis are illustrated.
whether enhancer complexes assembled with purified components can promote U2AF$_{65}$ cross-linking to the female-specific 3’ splice site and, if so, whether U2AF$_{35}$ is required. UV cross-linking studies were carried out by use of $^{32}$P-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 U2AF$_{65}$ 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 U2AF$_{35}$, however, resulted in U2AF$_{65}$ 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]. U2AF$_{65}$ 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 U2AF$_{65}$ 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 U2AF$_{65}$ cross-linking was observed in the experiments of Figure 5. However, this is likely caused by the relative efficiency of cross-linking of U2AF$_{65}$ 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 U2AF$_{65}$ 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, U2AF$_{35}$-dependent cross-linking of U2AF$_{65}$ 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, U2AF$_{35}$-dependent U2AF$_{65}$ 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 U2AF$_{65}$ binding.

Finally, we carried out UV cross-linking experiments to determine whether U2AF$_{35}$ is required for regulated (Tra- and Tra2-dependent) recognition of the 3’ splice
Figure 5. U2AF$^{35}$ is required for enhancer-dependent cross-linking of U2AF$^{55}$ to the female-specific 3' splice site. Effects of the other proteins on U2AF$^{55}$ cross-linking were examined at lower concentrations of U2AF$^{55}$. When all three proteins (U2AF$^{55}$, U2AF$^{35}$, and SR) are limiting, cross-linking of U2AF$^{55}$, but not of SR proteins, was observed. This occurs because the efficiency of cross-linking of SR proteins is less than that of U2AF$^{55}$. 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) U2AF$^{55}$ is required for constitutive enhancer-dependent U2AF$^{55}$ cross-linking. UV cross-linking was performed with the two RNA constructs dsxR2-5 and cfsxARE. (B) Distinct SR proteins are required for U2AF$^{35}$-dependent binding of U2AF$^{55}$ 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 U2AF$^{35}$-dependent U2AF$^{55}$ binding. The RNA used here is c/sxR1-5PRE.

Protein–protein interaction domains of U2AF$^{35}$ are required for enhancer-dependent splicing

To test the possibility that U2AF$^{35}$ functions as a bridge between U2AF$^{55}$ 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 U2AF$^{35}$ and U2AF$^{65}$ blocks their ability to reconstitute immunodepleted extracts. Amino acids 64–182 of U2AF$^{55}$ are required for its interaction with U2AF$^{65}$ (Zhang et al. 1992), whereas the SR domain of U2AF$^{35}$ is required for interactions with Tra, Tra2, and SR proteins [L. 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 U2AF$^{55}$, U2AF$^{35}$ΔSR 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, U2AF$^{55}$Δ35 [deletion of amino acids 95–138], which does not form a heterodimer with U2AF$^{55}$, cannot complement the splicing activity of U2AF$^{55}$ in immunodepleted extracts [Fig. 6A, lane 7].
The failure of the U2AF35Δ35 protein to promote enhancer-dependent splicing could be the result of its inability to interact with U2AF65. 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 and U2AF35 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 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 dsxRE 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 dsxRE, 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 dsxRE is located at its normal position, 300 nucleotides downstream from the 3’ splice site. Here, the SR proteins bound to the dsxRE 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.](genesdev.cshlp.org)
element in this model is the ability of U2AF to form a
bridge between U2AF and SR proteins bound to the
exon (Reed 1996). Similarly, U2AF 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 constitu­
tively spliced RNAs (Blencoe et al. 1994; O. Gozani
and R. Reed, pers. comm.). Thus, the constraint on exon
size may be a consequence of the requirement for forma­
tion 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 dsx RE. 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, how­
ever, 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 U2AF 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 sta­
ble 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 U2AF plays a critical role in both
constitutive and enhancer-dependent splicing by medi­
at ing essential protein–protein and protein–RNA in­
teractions required for accurate 3' splice site selection.

Materials and methods

Proteins and antibodies

The cDNAs containing the complete coding regions of U2AF (Zamore et al. 1992), U2AF (Zhang et al. 1992), and SRp40 (Screaton et al. 1995) were cloned into the baculovirus expres­
sion 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
RNAs were transcribed with T7 polymerase and capped during transcription. RNAs for gel filtration and splicing were labeled with \[^{32}P\]UTP.

In vitro splicing reactions were carried out as described in Tian and Maniatis (1992). In chase experiments, fractions containing ~1 ng of dsxR2-S RNA from either the E complex or the H complex were complemented with 8 µl of nuclear extract 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 µl of nuclear extract was used for functional assay in 25-µl 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 µl 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 \[^{32}P\]-labeled RNA (as specified in the figure legends). The reactions were then loaded directly onto a Sephacryl S-500 column (Pharmacia) (1.5 x 50 cm). The column was equilibrated and run with 20 mM Tris (pH 7.6), 20 mM DTT, and 2% SDS. The proteins were eluted from the column five times at room temperature, extracts were either frozen until needed. Depletion of U2AF was monitored by Western blotting with anti-U2AF antisera. After being passed through the columns five times at room temperature, extracts were used immediately for splicing reactions or frozen until needed.

**Immuno depletion 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-U2AF\(^{85}\) clonal 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-U2AF\(^{85}\) 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.

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