U1 snRNP targets an essential splicing factor, U2AF65, to the 3' splice site by a network of interactions spanning the exon

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A description of cellular factors that govern alternative splicing of pre-mRNA is largely incomplete. In the case of the rat preprotachykinin gene, splicing of the alternative exon E4 occurs by a poorly understood mechanism in which exon selection is under the positive control of U1 snRNP. Because the binding of U1 snRNP to the 5' splice site of E4 is coincident with the selection of the 3' splice site of E4, this mechanism would appear to involve interactions that bridge across the exon. In this work, a UV cross-linking strategy was used to identify possible RNA-protein interactions involved in the proposed exon-bridging model. Of particular interest is a prominent 61-kD protein, p61, that binds to the 3' splice site of E4 in a manner that is clearly facilitated by a downstream 5' splice site and U1 snRNP particles. The identity of p61 is the essential splicing factor U2AF65, on the basis of copurification and selective binding to polypyrimidine tracts. These results indicate a model in which exon selection is positively regulated by the communication of U1 snRNP and U2AF65. That is, a natural deficiency in binding U2AF65 to the 3' splice site that leads to exon skipping might be overcome by a mechanism in which U1 snRNP facilitates the binding of U2AF65 through a network of template-directed and exon-bridging interactions.

[Key Words: RNA-protein binding; U1 snRNP; U2AF65; alternative splicing; exon selection]

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Alternative splicing of pre-mRNA is a widespread process that is largely responsible for the diversity of polypeptides expressed in mammalian cells, yet a molecular understanding of the mechanisms and factors that govern this process is lacking. A central question of interest is how to explain the regulation of splice site selection that leads to the observed patterns of alternative 5', or 3' splice site selection, mutually exclusive exon selection, or controlled exon skipping (Andreadis et al. 1987; Smith et al. 1989; Maniatis 1991).

Our current understanding, from studies of single intron-containing pre-mRNA substrates, is that splice site recognition is achieved by the assembly of a spliceosome complex. The spliceosome is known to contain the small nuclear ribonucleoprotein (snRNP) particles U1, U2, U5, and U4 + U6, as well as additional protein factors (Padgett et al. 1986; Steitz et al. 1988; Green 1991; Guthrie 1991). In one of the earliest spliceosome assembly events, the 5' splice site at the upstream boundary of the intron is recognized by direct base-pairing with a 6- to 9-nucleotide stretch at the 5' end of U1 small nuclear RNA [snRNA] [Zhuang and Weiner 1986; Siliciano and Guthrie 1988; Rosbash and Seraphin 1991]. This RNA base-pairing interaction appears to be further stabilized by the involvement of a U1 snRNP-specific protein, U1-C (Heinrichs et al. 1990). Interestingly, engineered nucleotide changes in the 5' end of U1 snRNA have been shown to alter 5' splice site selection in vivo with a resulting shift in the pattern of splicing (Yuo and Weiner 1989). These results underscore the importance of U1 snRNP in 5' splice site selection and indicate its potential role in alternative splicing mechanisms.

Selection of the 3' splice site at the downstream boundary of the intron appears to be inherently more complex. This is the result of the number and arrangement of individual sequence elements that are involved in 3' splice site selection and also the number of factors capable of interacting with these sequences. In mammalian pre-mRNAs, the 3' splice site comprises an invariant AG dinucleotide positioned at the 3' splice site boundary, and this sequence is preceded almost invariably by a polypyrimidine tract, which can be quite variable in length and sequence. Farther upstream, a loosely conserved sequence element, termed the branch site, includes the adenosine, which is reacted in the first chemical step of splicing to form the branched nucleotide.

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Recognition of the branch site involves base-pairing of this sequence with an internal region of U2 snRNA. An established prerequisite for the branch site: U2 snRNP interaction is the binding of a protein, U2 auxiliary factor (U2AF), to the polypyrimidine tract. U2AF is a required splicing factor consisting of a large and a small protein subunit (Ruskin et al. 1988; Zamore and Green 1989, 1991).

In addition to U2AF, a number of distinct protein factors have been shown to bind with some degree of selectivity to the polypyrimidine tract and/or the AG dinucleotide. These include intron-binding protein, which appears to be associated with the U5 snRNP (Gerke and Steitz 1986; Tazi et al. 1986), heterogenous nuclear RNP (hnRNP) proteins A1, C1/C2, and D (Swanson and Dreyfuss 1988), and the polypyrimidine tract-binding (PTB) protein (Garcia-Blanco et al. 1989; Gil et al. 1991; Patton et al. 1991). Evidence for the importance of the hnRNP C1/C2 protein in RNA splicing is derived from experiments that show a disruption of splicing in vitro owing to specific immunodepletion (Choi et al. 1986). Splicing is also disrupted when PTB is depleted from a nuclear extract, and recovery of splicing activity is achieved by complementation with a PTB-enriched fraction (Patton et al. 1991). Further analysis will be needed, however, to determine the precise role of each of these protein factors.

One model to explain how alternative splice sites are selected is by a simple competition strategy. In this case, the relative strengths of the splice sites within a premRNA would be expected to be the primary characteristic determining the pattern of splicing and the level of products produced (Aebi et al. 1986; Eperon et al. 1986). Here, it is interesting to note that for pre-mRNAs with alternative 3′ splice sites, the protein splicing factor SF2/ASF has been shown to play a critical role in determining the selection of alternative 3′ splice sites (Ge and Manley 1990, Krainer et al. 1990). In these examples, the role of SF2/ASF appears to be antagonistic to the antagonistic role of the hnRNP A1 protein (Mayeda and Krainer 1992). Thus, an alteration in the relative levels of these two protein factors is capable of shifting the pattern of splicing, although the splice site sequences within the pre-mRNAs have not been altered.

In the case of the rat preprotachykinin gene, selection of the alternatively spliced exon E4 occurs by a mechanism that cannot be explained by a simple splice site competition model. A distinctive feature of this mechanism is that the 3′ splice site adjacent to E4 is inherently unreactive in its natural sequence context, even in the absence of a competing 3′ splice site. E4 selection is achieved only when an improved 3′ splice site is brought into proximity (downstream of E4) by a prior splicing event or by an engineered mutation that improves the base complementarity of the E4 5′ splice site and U1 snRNA (Nasim et al. 1990). Important clues to the role of the 5′ splice site in this mechanism have been indicated. First, improved binding of U1 snRNP at the 5′ splice site of E4 directly stimulates selection of the 3′ splice site of E4 [Grabowski et al. 1991; Kuo et al. 1991]. Second, the role of U1 snRNP binding at the downstream 5′ splice site adjacent to E4 can be attributed to a site-specific, threshold requirement that is not met by U1 snRNP binding to the upstream 5′ splice site (Grabowski et al. 1991). Together, these results indicate a model in which exon selection can be regulated positively by the binding of U1 snRNP to the 5′ splice site adjacent to the exon. Aside from substantial support for the role of U1 snRNP in this mechanism, the identification of other factors has been lacking.

The aim of the present study was to identify possible 3′ splice site factors that collaborate with U1 snRNP in facilitating exon selection. Here, we present evidence that a protein, p61, is recruited to the 3′ splice site of E4, under splicing conditions and in a manner that is facilitated by a downstream (consensus) 5′ splice site together with the U1 snRNP particle. The involvement of U1 snRNP in an exon-bridging model is indicated, as targeted binding of p61 is dependent on the 5′ terminus of U1 snRNA and the presence of U1 snRNP particles in the binding reaction. Using biochemical methods, we show that p61 is the large subunit of the essential splicing factor U2AF, U2AF65. The targeting of U2AF65 to the 3′ splice site by exon-bridging interactions may provide a sensitive mechanism that regulates exon selection in response to the strength of the downstream U1 snRNP : 5′ splice site interaction.

Results

Selective binding of p61 to 3′ half pre-mRNA substrates

The RNA substrates used to investigate RNA-protein interactions potentially involved in U1 snRNP-facilitated exon selection contained a single 3′ splice site with a portion of its associated exon (3′ half substrates; Fig. 1C). These substrates were designed to reveal protein binding that might be deficient for the 3′ splice site of E4 (3′–E4), compared with 3′ splice sites with strong splicing efficiency: the 3′ splice site region of exon 5 of the preprotachykinin gene (3′–E5), the adenovirus L2 exon (3′–Ad), and exon 3 of the rabbit β-globin gene (3′–BG). As expected, a variety of proteins in the nuclear extract were found to cross-link to all of the RNA substrates without apparent selectivity. However, one prominent cross-linked protein with an apparent molecular mass of 61.3 ± 0.8 kD, p61, was found to bind substrates containing strong 3′ splice sites under standard splicing conditions [Fig. 1A, lanes 2,4,5,10]. At reduced efficiency, a slightly faster migrating doublet is frequently associated with p61 binding for these RNAs. The protein doublet is distinct from p61 (see below). In contrast to the strong 3′ splice site containing substrates, only background levels of p61 binding was observed for the substrate containing the weak 3′ splice site of E4 [Fig. 1A, lanes 1, 6].

The question of particular interest was to determine what protein binding is enhanced by the binding of U1 snRNP to the downstream 5′ splice site of E4. Such factors found in this assay would be considered potential
candidates involved in the mechanism by which U1 snRNP facilitates splicing of the upstream intron. To focus on the role of the downstream, but not the upstream, 5’ splice site, protein binding was compared for a second set of substrates, which contained the entire E4 sequence with its adjacent 3’ and 5’ splice sites [Fig. 1C, 3’ + 5’ half substrates]. These substrates are essentially identical with the exception of nucleotide substitutions in the 5’ splice site that strengthen or weaken base complementarity to U1 snRNA.

These results clearly show selective binding of p61 to the substrate with the improved 5’ splice site, E4up, in contrast to analogous substrates with weaker 5’ splice sites, E4wt and E4dn [Fig. 1A, lanes 7–9]. Longer versions of these RNAs that are functional splicing substrates also show selective binding of p61 but with much higher levels of background protein binding [data not shown]. For the studies presented here, shortened substrates were used to maximize p61 binding. Densitometry scanning of duplicate reactions, which was used to quantify the enhancement of binding of p61 to the E4up compared with the E4dn substrate, routinely shows a 2–3 × ± 0.1 × higher binding of p61 to the E4up substrate. For the experiment of Figure 1A, this ratio is 2.5 ×.

The specificity of p61 binding to substrate E4up is indicated by the effect of competitor RNAs [Fig. 1B]. A substantial decrease in p61 binding to the [32P]-labeled substrate, E4up, is shown by the addition of unlabeled substrates containing strong 3’ splice sites, 3’-E5, 3’-Ad, and 3’-BG [Fig. 1B, lanes 5–7, 12–14, and 15–17]. Even at the lowest level of competitor added [5 × molar excess], a substantial reduction of p61 binding is observed for these three competitor RNAs. In contrast, the competitor that contains the 3’ splice site of E4 [without the downstream 5’ splice site] shows no reduction in p61 binding even at the highest level of competitor added, 25 × molar excess [Fig. 1B, lanes 2–4]. Therefore, we conclude that the binding of p61 to a substrate with a weak polypyrimidine tract is facilitated by a downstream 5’ splice site, but for substrates with consensus polypyrimidine tracts, p61
U1 snRNP targeted binding of U2AF65

binding occurs in the absence of a downstream 5' splice site.

**p61 binding is localized to the polypyrimidine tract and AG dinucleotide**

To localize sequence elements important for p61 binding within the RNA substrate, we used a strategy that combined mutational analysis and differential nucleotide labeling. Two regions of the 3' splice site of E4up, the polypyrimidine tract and the AG dinucleotide, were destroyed by mutation to test the importance of these sequences for p61 binding. If p61 is targeted specifically to the 3' splice site region by U1 snRNP, then mutations in the 3' splice site region would be expected to disrupt binding of the protein.

A mutation that destroyed the polypyrimidine tract consisted of changing 5 uracil residues to adenosines between the branch site and the AG dinucleotide (substrate E4py; Fig. 1C). A reduction in p61 cross-linking to near background levels was observed for the polypyrimidine tract mutant, E4py, compared with the substrate with the wild-type polypyrimidine tract, E4up, under conditions where the substrates were labeled with \[^{32}P\]UTP (Fig. 2A, lanes 1,4). To directly compare p61 binding, \[^{32}P\]GTP and \[^{32}P\]CTP were used to label the invariant nucleotides. When CTP-labeled substrates were used, the binding of p61 was completely lost for the E4py compared with the E4up substrates (Fig. 2A, lanes 1,3). Because the polypyrimidine tract mutation is the only difference between these substrates and invariant C residues were uniformly labeled, these results show that p61 cross-linking is completely disrupted by the polypyrimidine tract mutation (Fig. 2B). This experiment also indicates that in general, protein cross-linking to U residues is preferred (Fig. 2A, lanes 14–21). However, as there are many U residues distributed throughout the E4up and E4py substrates, nonspecific p61 cross-linking, or cross-linking at a site other than the polypyrimidine tract would not have been expected to abolish the cross-linking signal when the E4py substrate was labeled at invariant cytosine residues. Thus, p61 must be closely associated with the polypyrimidine tract.

One cautionary note is that a loss of UV cross-linking does not necessarily mean a complete loss of RNA–protein association. However, it is important to note that the results of the competition experiment shown in Figure 1B strongly support the conclusion that p61 binds to the polypyrimidine tract, as three unrelated substrate RNAs with consensus polypyrimidine tracts (3'-E5, 3'-BG, and 3'-Ad) effectively compete for binding of p61 to the E4up substrate, whereas a substrate with a weak polypyrimidine tract (3'-E4) does not compete for binding.

To examine the importance of the 3' splice site AG dinucleotide for p61 binding, the dinucleotide CU was substituted at this position to produce the new mutant, E4CU (Fig. 1C). This mutation was found to reduce (but not abolish) binding of p61 compared with the substrate with the intact AG (Fig. 2A, lanes 1,3). In a similar fashion, a 3' half substrate with an AG → CU mutation, 3'-E5CU, was also found to have reduced binding of p61 (Fig. 1A, lanes 2,3). Consistent with the above results, the 3'-E5CU mutant is a weak competitor for p61 bind-

**Figure 2. Localization of p61 binding to the 3' splice site by mutational analysis and differential nucleotide labeling.** (A) Substrates (top) individually labeled with \[^{32}P\]ATP, \[^{32}P\]CTP, \[^{32}P\]GTP, or \[^{32}P\]UTP were tested in the UV cross-linking assay as in Fig. 1A. 3' Splice site mutations are indicated schematically in Fig. 1C; sequences are specified in Table 1. (B) Comparison of polypyrimidine tract sequences for substrates labeled with CTP or UTP. Commas demarcate RNA fragments generated by ribonuclease A cleavage. The conditions under which ribonuclease digestion is performed result in complete digestion of the RNA sample. Labeled phosphates are indicated by dots. The boxed region encompasses the polypyrimidine tract region, in which U → A mutations (underlined) result in a loss of p61 binding when CTP is used to label the invariant nucleotides. Approximate relative binding of p61 is indicated [right].
Targeted binding of p61 is governed by U1 snRNP

Because the binding of p61 to a weak polypyrimidine tract is clearly facilitated by a downstream, consensus 5' splice site, the involvement of the U1 snRNP particle is indicated. Up to this point these data show that binding of p61 to the substrate E4up requires a combination of sequence elements located at two distinct positions (in cis) in the RNA substrate: the polypyrimidine tract/AG dinucleotide at the 3' splice site, and the downstream 5' splice site. To further investigate the role of the 5' splice site in the binding of p61, we tested the likely possibility that this sequence interacts with U1 snRNP. Three specific expectations were subjected to experimental tests: [1] The 5' terminus of U1 snRNA should be required for the binding of p61; [2] stable complex formation between U1 snRNP and the E4up substrate should be evident; and [3] removal of U1 snRNP particles from the nuclear extract should abolish p61 binding.

To assess the role of the 5' terminus of U1 snRNA, the nuclear extract used for the p61-binding reaction was pre-treated with RNase H and an oligonucleotide complimentary to nucleotides 1-13 of U1 snRNA. These results show that p61 binding was reduced to background levels in the extract in which the 5' terminus of U1 snRNA was quantitatively destroyed [Fig. 3A, lane 3]. In contrast, control reactions, which destroyed either U2 or U6 snRNA did not affect binding of p61 to the same substrate [Fig. 3A, lanes 4,5]. RNA analysis verifies that the RNase H digestion resulted in specific and quantitative cleavage of the individual snRNAs [Fig. 3A, right). Thus, we conclude that the 5' terminus of U1 snRNA is required for targeted binding of p61 to the E4up substrate.

To examine stable complex formation with U1 snRNP, native gel electrophoresis was used in combination with RNase H treatment of nuclear extracts [Fig. 3B]. In the untreated nuclear extract, a stable U1 snRNP-substrate complex is clearly evident for the E4up substrate, and formation of this complex is circumvented by prior cleavage of the 5' terminus of U1 snRNA [Fig. 3B, lanes 1,2]. In addition, the U1 snRNP-E4up substrate complex is efficiently formed in nuclear extracts in which U2 or U6 snRNA has been cleaved [Fig. 3B, lanes 3,4]. Cleavage with the U2- and U6-specific oligonucleotides enhances the formation of the U1 snRNP-substrate complex most likely because this treatment releases U1 snRNP from large complexes normally present in the nuclear extract. In contrast to the results observed for the E4up substrate, substrate E4dn, which lacks a 5' splice site but is otherwise identical to E4up, does not form a stable U1 snRNP complex under these p61-binding conditions [Fig. 3B, lanes 6-9].

Interestingly, the E4py substrate forms a stable complex with U1 snRNP [Fig. 3B, lanes 11-14]. The E4py substrate contains a polypyrimidine tract mutation that
disrupts UV cross-linking of p61 [see above, Figure 2] but is otherwise identical to the E4up substrate. These results are significant, in that they show that the binding of U1 snRNP to the RNA substrate does not by itself result in p61 cross-linking when the polypyrimidine tract is missing. In sum, the experiments of Figures 1–3 indicate that the distinct binding sites for p61 and U1 snRNP must both be present on the RNA substrate for targeted binding of p61 to occur.

To directly assess the involvement of the U1 snRNP particle in the p61-binding interaction at the polypyrimidine tract, a complementation assay was performed using a U1 snRNP-depleted nuclear extract plus an enriched U1 snRNP fraction. If p61 binding is truly dependent on binding of U1 snRNP to the downstream 5' splice site, then binding of p61 should be disrupted in the U1-depleted extract but recovered upon the addition of U1 snRNP particles.

**Figure 4.** The U1 snRNP particle is required for targeted binding of p61. (A) The E4up substrate, UTP labeled, was used to assay p61 binding as in Fig. 1A. HeLa nuclear extracts include untreated [NE], U1 depleted [ΔU1], or U2 depleted [ΔU2] and the U1 snRNP-enriched fraction [U1]. The untreated nuclear extract was diluted by a factor of 3, [lanes 1,7] to match the protein concentration of the U1-depleted extract [lane 2]. Identical amounts of the U1-enriched fraction were used in reactions in lanes 3 and 4. The reaction in lane 6 was performed by combining equal amounts of the U1- and U2-depleted extracts. (B) Northern blot analysis of the DEAE-purification column in which the U1 snRNP-enriched fraction [fraction 18; arrow] was obtained for the experiment in A. The starting material [IN] loaded onto the column contained pooled fractions from a CsCl gradient enriched for U1 snRNP. Peaks from the DEAE column [numbered fractions] or the column flow through [FT] are indicated [top]. Equivalent volumes of each peak were deproteinized, separated on a 10% polyacrylamide/7 M urea gel, blotted onto a nylon membrane, and probed for U1, U2, U4, U5, and U6 snRNAs as described [Kuo et al. 1991]. (C) Stoichiometry of exogenously added U1 snRNP to endogenous U2 snRNP in the complementation assay. UV cross-linking with the E4up substrate was used to monitor p61 binding in the U1-depleted extract, without [lane 2] or with 1, 3, 5, or 7 μl of U1-enriched fraction added back [lanes 3–6]. A control reaction, in which 7 μl of the U1-enriched fraction alone is used, shows background levels of p61 binding. Control reactions showing normal levels of p61 binding include untreated nuclear extract [lane 7] or equivalent amounts of a U2-depleted extract [lane 8]. The relative amounts of p61 binding were quantified by densitometry scanning of appropriate exposures of the autoradiograph shown and are expressed as a percent of p61 binding obtained in the untreated nuclear extract. For the reactions in lanes 3–6, substrate RNA is present in molar excess of U1 snRNP added back. (D) Northern blot analysis of U1 and U2 RNAs was used to estimate the relative levels of these RNAs in the complementation assay [lanes 1–5] by comparison to endogenous levels of these RNAs in the untreated nuclear extract [lanes 6–10]. Amounts [μl] of the U1-enriched fraction added back are indicated at the top of lanes 1–5; fold dilutions of the untreated nuclear extract are indicated at the top of lanes 6–10.
Binding of p61 was clearly disrupted when UV cross-linking was performed with the U1-depleted extract, whereas addition of the U1 snRNP-enriched fraction restored binding of p61 [Fig. 4A, lanes 1–3]. Northern blot analysis verifies that U1 snRNP was quantitatively depleted [Fig. 4D, lane 1]. Importantly, the observation that the U2 snRNP-depleted control retains efficient binding of p61 shows that the depletion procedure itself does not remove p61 from the extract [Fig. 4A, lane 5]. Because the experiments presented below demonstrate that p61 is identical to the 65-kD subunit of U2 auxiliary factor [U2AF65], Western blot analysis with a U2AF65-specific antibody [PepA] was performed to assess the levels of U2AF in the depleted extracts. These results verified that the untreated extract and the U1 and U2 snRNP-depleted extracts contained equivalent levels of U2AF65, but no U2AF65 was detectable in the U1 snRNP-enriched fraction [data not shown]. Owing to the greater sensitivity of the UV cross-linking assay, however, the U1 snRNP-enriched fraction showed background levels of p61 cross-linking, which indicates that there is trace contamination of p61 in this fraction [Fig. 4A, lane 4].

U1 snRNP or U2 snRNP was selectively and quantitatively depleted from these extracts by use of 2’-O methyl oligoribonucleotides complementary to the 5’ ends of the snRNAs; these extracts were gifts of A. Lamond [Barabino et al. 1990]. The U1 snRNP-enriched fraction was prepared in two steps: First, a concentrated U snRNP fraction was obtained by isopycnic centrifugation of a HeLa nuclear extract in cesium chloride at 15 mm magnesium [Lelay-Taha et al. 1986], second, pooled fractions containing U1 snRNP were loaded onto a DEAE column and eluted with a linear gradient of ammonium chloride. Fraction 18, which was used for the complementation assay, contained concentrated U1 snRNP and was devoid of the other U snRNPs, with the exception of a trace level of U6 [Fig. 4B]. The functionality of the U1 snRNP-enriched fraction was determined by testing the U1 snRNP-depleted extract for its ability to splice a single intron-containing pre-mRNA derived from adenovirus in the presence and absence of U1 snRNP added back. The U1-depleted extract was found to be devoid of splicing activity, whereas complementation with the U1-enriched fraction restored splicing, although inefficiently [data not shown].

The stoichiometry of exogenously added U1 snRNP relative to endogenous U2 snRNP was measured to assess the complementation of the U1 snRNP-enriched fraction used for complementation of p61 binding. Within a sevenfold range of U1 snRNP added back to the U1-depleted extract, binding of p61 was recovered to nearly the level of the untreated nuclear extract [Fig. 4C, lanes 1–6]. In equivalent reactions, the relative amount of U1 snRNP added back was compared with the endogenous U2 snRNP by Northern blot analysis [Fig. 4D, lanes 1–5]. These results show that the level of U1 snRNP that restores p61 binding essentially restores the ratio of U1 to U2 snRNP normally found in the nuclear extract [Fig. 4D, see e.g. lanes 3,7]. At this level of added U1 snRNP, p61-targeted binding is saturated, which indicates that a factor other than U1 snRNP is limiting in the nuclear extract.

p61 is U2AF65

Because p61 is a candidate for a required splicing factor involved in U1 snRNP-facilitated 3’ splice site selection, it is of interest to establish the identity of p61 and determine its functional role in splicing. First we asked whether p61 is related to one of the polyuridylic tract binding proteins that have been characterized previously. Within this category, two proteins were judged to be especially likely candidates because their apparent molecular masses are close to that of p61: the 62-kD PTB protein and U2AF65 [Garcia-Blanco et al. 1989; Zamore and Green 1989].

The experimental approach used to pursue the identity of p61 was to fractionate the nuclear extract under conditions known to separate the PTB and U2AF activities, followed by additional steps leading to the purification of U2AF [Fig. 5A; Zamore and Green 1989]. If p61 is identical to one of these factors, then copurification should be evident. In contrast, if p61 is unique, a distinct purification pattern should be observed. To track the course of each factor in this purification scheme, UV cross-linking was used to assay for p61 binding, whereas Western blot analysis was used to detect PTB and U2AF. To simplify the analysis, the 3’-BG substrate was used in the UV cross-linking assay, as p61 binding occurs efficiently to this substrate and binding is independent of U1 snRNP [see below]. It is relevant to note that for technical reasons immunoprecipitation analysis could not be used to identify p61 definitively.

Copurification of p61 and U2AF65 is clearly evident throughout the course of the fractionation scheme. This is most apparent in the second and third column steps, as these fractions exclusively contain p61 binding activity [Fig. 5B, lanes 8,11], and these are the only fractions that contain U2AF65 [Fig. 5C, x PepA]. Furthermore, these fractions are ultimately derived from a fraction [0.25 M DEAE], that contains U2AF but not PTB [Fig. 5C, x PepA and x PBP].

The most highly purified sample of U2AF used for the experiment above [three-column purified] was subjected to SDS-PAGE analysis and protein staining [Fig. 5D]. These results show that the 65- and 35-kD subunits of U2AF are the predominant components in that fraction. It is not clear why there is less protein staining of the 35-kD subunit compared to the 65-kD subunit in the purified U2AF sample. Importantly, the 65-kD protein subunit aligns exactly with the protein band that stains with the PepA antibody by Western blot analysis [Fig. 5C, lane 2MGu]. Thus, copurification through three chromatographic steps provides strong evidence that p61 is identical to U2AF65.

It is of interest to note that U1 snRNP separates from U2AF in the second column step [0.5 m heparin], and is therefore absent from the second and third column fractions that exclusively contain p61-binding activity [Fig. 5C, x 7-13]. Because the chromatographic behavior of the U1–C polypeptide exactly matches that of the U1–70K
U1 snRNP targeted binding of U2AF65

Figure 5. p61-binding activity copurifies with U2AF65 through three chromatographic steps. (A) Scheme for separation of U2AF and PTB by DEAE column chromatography followed by purification of U2AF on heparin–agarose, and poly(U)-Sepharose columns as described (Zamore and Green 1989). Boxes indicate copurification of p61 and U2AF and separation of PTB and U1 snRNP before the poly(U)–Sepharose column based on the assays shown in B and C. (PTB) Poly-pyrimidine tract binding protein; (U1) U1 snRNP; (U2AF) U2 auxiliary factor. (B) UV cross-linking assay for p61-binding activity. The 3'-BG substrate was used to assay for p61-binding activity. UV cross-linking was performed with equivalent volumes of each fraction as in Fig. 1A. Column fractions are indicated (top). [NE] Unfractionated HeLa nuclear extract; [2MGu] Guanidine-HCl elution of poly(U)-Sepharose column. Substrate B3P3 was cross-linked in the presence of untreated nuclear extract and serves as a marker for the PTB protein doublet (lane 1). [C] Western blot analysis used to track U2AF, PTB, and U1 snRNP. Aliquots of column fractions (indicated at top) used for the experiment in B were separated on a 12.5% SDS-polyacrylamide gel, as in B and transferred to nitrocellulose. Primary antibodies were PepA, U2AF65-specific; PBP-specific; 7-13, U1 snRNP–C protein specific. A polypeptide of apparent molecular mass 74 kD, which is detected by the PepA antibody, was separated from U2AF65 on the heparin–agarose column and was not investigated further (heparin 0.5 M). (D) SDS-PAGE analysis of purified U2AF. An aliquot of three-column-purified U2AF from the sample shown in lane 2MGu in C was resolved on a 12.5% polyacrylamide gel, and proteins were detected by silver staining. The large (65) and small (35) subunits of U2AF are indicated at left. Molecular mass markers (Bio-Rad) (lanes M1 and M2) are identical samples, except that M2 contains five times more material than lane M1. Apparent molecular masses (in kD) are indicated at right. It should be noted that the apparent molecular mass of p61 in its cross-linked form (61.3 ± 0.8 kD) is experimentally indistinguishable from that of the highly purified (and uncross-linked) U2AF65, as measured in our hands by Western blot analysis or total protein staining (60.1 ± 0.7 kD). These apparent molecular masses are based on a minimum of six independent measurements.

polypeptide (data not shown), we interpret these results to indicate the behavior of the intact U1 snRNP particle. These results demonstrate that p61 binding to the 3'-BG substrate occurs in a U1 snRNP-independent manner under these conditions (nonfacilitated binding).

To obtain confirmatory evidence that p61 is U2AF65, a second approach was used in which U2AF was selectively and quantitatively depleted from the nuclear extract using affinity chromatography on poly(U)–Sepharose (Fig. 6A, Zamore and Green 1989). As anticipated, p61 binding to the 3'-BG substrate was lost in the U2AF-depleted extract and recovered exclusively in the U2AF-enriched fraction (Fig. 6B, lanes 2 and 3, respectively). Binding of p61 was also restored with alternative sources of U2AF (Fig. 6B, lanes 4–6). Similarly, binding of p61 to the E4 up substrate was lost in the U2AF-depleted extract (Fig. 6C, cf. lanes 1 and 3). In sum, these data, combined with the copurification results shown above, provide compelling evidence that p61 is U2AF65.

Binding of U2AF65 and the PTB protein can be distinguished

It is apparent that in the U2AF-depleted extract the pat-
Figure 6. Depletion and recovery of p61-binding activity tracks with U2AF65. [A] Scheme for preparation of U2AF-depleted and U2AF-enriched extracts and detection of U2AF65 in these extracts by Western blot analysis using the PepA antibody. [B] UV cross-linking was used to assay for p61 binding to substrate 3’-BG using a U2AF-depleted extract (ΔU2AF) or a 2 M guanidine fraction enriched in U2AF (2MGu). These fractions were prepared by chromatography of a HeLa nuclear extract on poly(U)-Sepharose, as shown schematically in A, using the procedure described by Zamore and Green (1989). Control reactions contained uridincated nuclear extract (NE). Complementation of p61-binding activity using the U2AF-depleted extract plus the U2AF-enriched fractions (2MGu, and 1MHep or purified U2AF (U2AF) is shown (lanes 4–6). Complementation with buffer alone is indicated (–). The protein doublet that migrates slightly faster than the position of p61 is the PTB protein. [C] Same as B but with the E4up and E4dn substrates. U2AF, three-column-purified U2AF from the experiment in Fig. 5. [S-100] Cytoplasmic extract from HeLa cells. Binding reactions of lanes 17–22 contained three components: component 1, U2AF; component 2, U1 snRNP; and component 3, a U2AF-depleted extract that was digested with (XMN) or without (Xmock) micrococcal nuclease, or not treated, (XNT). For the experiment shown in the right panel, the 1 M heparin fraction from the experiment in Fig. 5C was used as the source of U2AF65. This fraction is devoid of U1 snRNP and behaves identically to the three-column-purified U2AF sample. All other abbreviations are identical to that of B. UV cross-linking to the PTB protein doublet is evident in lanes 3 and 4 and in lanes 17–22 (see Fig. 5B).

Interm of protein binding changes substantially. Interestingly, a protein doublet that migrates slightly faster than p61/U2AF65 is strongly enhanced (Fig. 6B, lanes 1,2). This protein doublet is the PTB protein, based on the following evidence. (1) This protein doublet, which is also evident in the crude nuclear extract, comigrates with the predominant species that binds to the B3P3 substrate, which has been shown previously to bind almost exclusively to PTB (Fig. 5B, lane 1,2, Mullen et al. 1991). (2) This protein doublet fractionates as expected for PTB (Garcia-Blanco et al. 1989). It is eluted exclusively in the 0.15 M fraction of the DEAE column, where the PTB antigen is also exclusively found (Fig. 5B, lane 3; Fig. 5C, x PBP). In addition, chromatography of this protein doublet on heparin–agarose matches the expected behavior of PTB (data not shown).

PTB binding is clearly evident to all of the 3’ half substrates tested in this assay that have strong polypyrimidine tracts, and its binding is unaffected by an AG → CU mutation at the 3’ splice site (Fig. 1A, lanes 2–5). This lack of sensitivity to the AG dinucleotide is a normal property of PTB binding (Garcia-Blanco et al. 1989). Interestingly, under the conditions used in this assay (standard splicing conditions), U2AF65 binding routinely predominates over PTB binding in the crude nuclear extract, and PTB binding appears to be unmasked when U2AF is depleted from the extract (Fig. 6B, lanes 1,2).

In contrast to the substrates with strong 3’ splice sites, the E4-containing substrates show essentially no binding to PTB, including the E4up substrate, which contains a downstream (consensus) 5’ splice site (Fig. 6C, lanes 1,9,15; see also Fig. 1A, lanes 1,6–9). However, when U2AF is depleted from the extract, PTB binding to the E4up and E4dn substrates is evident, although this binding is not selective for the E4up substrate (Fig. 6C, lanes 3,4).

Fractions enriched in U2AF65 and U1 snRNP are not sufficient to reconstitute U1 snRNP-targeted protein binding: evidence for a third component

Because p61/U2AF65 binding to the E4up substrate is
facilitated by U1 snRNP, we asked whether the selectivity of this binding interaction could be reconstituted with fractions enriched in U2AF65 and U1 snRNP. To evaluate U1 snRNP-facilitated protein binding (targeted binding), the substrates with and without a downstream 5' splice site, E4up and E4dn, respectively, were tested in parallel in the UV cross-linking assay using U2AF-depleted and U2AF-enriched fractions. U1 snRNP is found exclusively in the U2AF-depleted extract, and U2AF is found exclusively in the U2AF-enriched extract.

As expected, when the U2AF-depleted extract was used as the protein source, binding of p61/U2AF65 was lost, even though this extract contains U1 snRNP (Fig. 6C, lanes 3,4). Similarly, when the reciprocal fraction that is enriched in U2AF, but lacking U1 snRNP, was used as the protein source, p61/U2AF65 binding was recovered, but it was not selective for the E4up substrate (Fig. 6C, lanes 5,6). However, targeted binding was reconstituted when the U2AF-depleted and U2AF-enriched fractions were recombined (Fig. 6C, lanes 7,8). These results show 1.7 ± 0.1 higher binding of p61/U2AF65 for the E4up compared with the E4dn substrate. In this case, the reconstitution of targeted binding is less efficient than control reactions containing unfractionated nuclear extract (Fig. 6C, lanes 1,2,9,10; 2.4 ± 0.1 x).

Interestingly, U1 snRNP-facilitated binding of p61/U2AF65 was not reconstituted with a highly purified source of U2AF65 and the U1 snRNP-enriched fraction (Fig. 6C, lanes 13,14). Equivalent binding of p61/U2AF65 to the E4up and E4dn substrates is observed under these conditions, and these results show cross-linking to the p61/U2AF65 protein alone. When each of these fractions is titrated in the binding reaction, similar results are observed indicating that equivalent binding is not simply an artifact of the relative concentration of U1 snRNP to U2AF in the binding reactions (data not shown). This unexpected result is consistent with two possible explanations: (1) the inactivation of U1 snRNP and/or U2AF activities, or (2) the lack of additional required components. Although we cannot fully discount the former possibility, it appears unlikely as the U1 snRNP and U2AF fractions are capable of restoring splicing activity to U1 snRNP- and U2AF-depleted extracts, respectively (data not shown).

The cytoplasmic S-100 extract, which was tested as a control in this experiment, shows equivalent cross-linking of a variety of proteins to the E4up and E4dn substrates but essentially no cross-linking to p61/U2AF65 (Fig. 6C, lanes 11,12). Although the S-100 extract contains moderate levels of U1 snRNP and low levels of U2AF65, as determined by Western blot analysis, these results suggest that the composition of the S-100 extract is not compatible with U1 snRNP targeting of p61/U2AF65.

If U2AF and U1 snRNP alone are unable to reconstitute targeted binding to the E4up substrate and these two activities are demonstrated to be functional, then the requirement for an additional activity is indicated. An additional activity might be a direct requirement for exon bridging or might (indirectly) be necessary to establish an appropriate context for targeted binding. To gain positive evidence for this putative activity, we searched for a biochemical fraction devoid of U2AF and U1 snRNP that would reconstitute targeted binding of p61/U2AF65 in the presence of fractions containing these two known components. The right-hand panel of Figure 6C shows an example of such an experiment.

We prepared a crude biochemical fraction devoid of U2AF and U1 snRNP by extensively treating the U2AF-depleted extract with micrococcal nuclease to destroy the U1 snRNP contained in that fraction. Micrococcal nuclease treatment was performed as described previously, and the enzyme was subsequently inactivated by the addition of EGTA to chelate the required calcium ions (Krainer and Maniatis 1985). The U2AF-depleted extract treated with micrococcal nuclease (ΔU2AF × MN), was used as the third component (component 3) in the reconstitution assay because it was devoid of U2AF and U1 snRNP. When component 3 was combined with U2AF (component 1) and U1 snRNP (component 2), targeted binding of p61/U2AF65 was reconstituted (Fig. 6C, lanes 21,22). Control reactions in which component 3 consisted of untreated or mock-treated extract resulted in a similar efficiency of targeted binding (Fig. 6C, lanes 17–20). For the reactions of Figure 6C, lanes 17–22, the mean ratio of p61 binding to the E4up and E4dn substrates is 2.2 ± 0.1 x. In the absence of component 3, equivalent binding of p61/U2AF65 to the E4up and E4dn substrates is routinely observed (see e.g., Fig. 6C, lanes 13–14). These results are consistent with the idea that the U2AF-depleted fraction contains U1 snRNP and additional micrococcal nuclease-resistant factors that are required for targeted binding of p61/U2AF65. Unequivocal demonstration of an additional activity, however, must await purification and identification of the active components in fraction 3.

Discussion

In this work we show that the essential splicing factor, U2AF65, is targeted to the 3' splice site of an alternatively spliced exon in a manner that is facilitated by U1 snRNP and the downstream 5' splice site. These results provide evidence for a network of interactions spanning the exon that might provide the underlying structural foundation for a model of alternative splicing whereby exon selection is under the positive control of U1 snRNP (Fig. 7).

Model for an exon-bridging network

Evidence for the involvement of U1 snRNP and U2AF65 in an exon-bridging network is compelling. (1) Binding of p61/U2AF65, as detected by UV cross-linking, is dependent on the presence of a downstream (consensus) 5' splice site, and U1 snRNP particles must be present in the binding reaction. Furthermore, the 5' terminus of U1 snRNA is required for the association of both U1 snRNP and p61/U2AF65 with the RNA substrate. (2) The location of p61/U2AF65 binding must encompass the 3'
Figure 7. Model for a network of interactions bridging the exon. Strategies for 3’ splice site selection are shown for a strong [I] or a weak [II] polypyrimidine tract, in which the sequences are examples derived from the rabbit β-globin (3’-BG] and the rat preprotrychynkinin exon 4 [E4up] substrates, respectively (see Discussion). U residues in the polypyrimidine tracts are outlined for comparison. Question marks indicate possible factors that may be needed in addition to those shown. (p61/U2AF65) U2AF65, [lines] intron segments, [boxes] exons, [3’ ss] 3’ splice site; [5’ ss] 5’ splice site; [5'] 5’ end of U1 snRNA.

splice site, as UV cross-linking is disrupted by both mutations that destroy the polypyrimidine tract or AG di-nucleotide and by the addition of stoichiometric levels of unlabeled competitor RNAs that contain strong 3’ splice sites. Furthermore, the importance of an intact 3' splice site is strongly indicated, as U1 snRNP binding to the 5’ splice site is not sufficient to target p61/U2AF65 binding when the upstream polypyrimidine tract has been destroyed by mutation [substrate E4py]. [3] The identity of p61/U2AF65 was established using a biochemical strategy that quantitatively separates U2AF from PTB. Purification of p61/U2AF65 was achieved by a three-step chromatography procedure that has been shown previously to purify U2AF to homogeneity (Zamore and Green 1991). Purified p61/U2AF65 contains two prominent polypeptides corresponding to the large (65 kD) and small (35 kD) subunits of U2AF.

The specificity for U2AF65 in the targeting model is strongly indicated by the observation that PTB is not targeted by U1 snRNP, even though PTB has a high affinity for polypyrimidine tracts, similar to U2AF65, and is available for binding in the nuclear extract. Even when U2AF is removed from the nuclear extract by chromatography on poly(U)-Sepharose, PTB binding is clearly detected (Fig. 6). U1 snRNP targeting appears to occur exclusively with U2AF65.

The outcome of this work provides evidence for a structural model in which a network of interactions involved in targeting U2AF65 binding comprises two template-directed contacts and exon-bridging interactions. These include A, the base-pairing of U1 snRNA to the downstream 5’ splice site, B, the interaction of U1 snRNP and U2AF65, through direct or indirect contact across the exon, and C, the interaction of U2AF65 with the polypyrimidine tract of the upstream 3’ splice site (Fig. 7). In this model U1 snRNP binding to the 5’ splice site is believed to initiate the binding of U2AF65 to the [weak] polypyrimidine tract located upstream of the alternatively spliced exon E4. It is likely that splicing would be activated as a natural consequence of "loading" U2AF65 at this 3’ splice site, as the U2AF65 polypeptide is a required splicing factor (Zamore et al. 1992). An activation of splicing to this 3’ splice site is achieved when U1 snRNP is loaded specifically at the downstream, but not the upstream, 5’ splice site (Grabowski et al. 1991).

It is interesting to note that U2AF65 was found to bind readily to RNA substrates with highly active 3’ splice sites in the absence of a 5’ splice site and U1 snRNP particles. Each of these highly active 3’ splice sites contains a consensus polypyrimidine tract in contrast with the polypyrimidine tract of the E4 3’ splice site, which is shorter and interrupted by purines [Table 1]. These results are consistent with the known affinity of U2AF65 for polypyrimidine tracts and also consistent with the disruptive effects of polypyrimidine tract mutations on splicing activity [Fu et al. 1988; Reed 1989; Smith and Nadal-Ginard 1989; Mullen et al. 1991]. Thus, the RNA-binding studies presented here show that under in vitro splicing conditions U1 snRNP is not needed to facilitate U2AF65 binding to 3’ splice site sequences that are inherently strong, whereas for the alternatively spliced E4, U2AF65 binds only with the assistance of U1 snRNP. In more general terms, these results provide evidence for a sensitive on/off mechanism of exon selection that would appear to be based, ultimately, on U2AF65 binding. For an alternatively spliced exon like E4, inefficient binding of U2AF65 would be the predicted driving force for exon skipping [off], whereas U1 snRNP-facilitated U2AF65 binding would be the predicted driving force for exon selection [on].

Possible alternatives to the exon-bridging model, described in Figure 7, have been considered and judged to be unlikely. One of these involves the proposed targeting of U2AF65 to the RNA substrate by interaction with free [i.e., nontemplate bound] U1 snRNP. This model is considered unlikely because the data shown in this paper demonstrate that targeted p61/U2AF65 binding requires the 5’ terminus of U1 snRNA as well as the presence of a consensus 5’ splice site sequence in the RNA template. If targeted binding to the RNA substrate was the result of interaction with a free U1 snRNP particle, then cleavage of the 5’ terminus of U1 snRNA would not have been expected to disrupt p61/U2AF65 binding. It is relevant.

Table 1. 3’ Splice site sequences

| 3’ Splice site sequences | E4wt | E4py | E4CU | 3’-E5 | 3’-ESC | 3’-Ad | 3’-BG |
|-------------------------|------|------|------|-------|--------|-------|-------|
|                         | CAA  | -A   | AUC  | GGC   | UGU    | UCC   | UGCC  |
|                         | UUC  | -A   | AUC  | UCU   | UGU    | UCU   | UCG   |
|                         | AUC  | -A   | AUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UCU  | -A   | AUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UGU  | -C   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UCU  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |
|                         | UUC  | -U   | UUC  | UGU   | UGU    | UCU   | UCU   |

The colon, 3’ splice site, mutations, underlines, and dashes indicate identity with the nucleotide immediately above.
to note that U1 snRNP-targeted binding of p61/U2AF65 shown in this study is clearly different from the SC35-mediated interaction of U1 snRNP at the 3' splice site that has been described previously by Fu and Maniatis (1992). The SC35-mediated U1 snRNP interaction with the 3' splice site does not require the 5' terminus of U1 snRNA or a 5' splice site.

Another possible model involves the proposed interaction of the U2AF65 protein and the U1 snRNP particle through trans-acting interactions involving two separate RNA template strands: one in which U2AF65 is bound to the polypyrimidine tract/AG dinucleotide, and one in which the U1 snRNP particle is bound to the 5' splice site. This model was tested by performing mixed substrate experiments in which a radiolabeled substrate RNA containing a weak 3' splice site, but lacking a downstream 5' splice site, was incubated with an excess of unlabeled substrate RNA containing a consensus 5' splice site. These results show that a consensus 5' splice site located on a separate RNA strand fails to target p61/U2AF65 binding to the labeled RNA substrate under standard p61/U2AF65-binding conditions (B.E. Hoffman and P.J. Grabowski, unpubl.). Thus, a model in which U1 snRNP and U2AF65 interact with one another when bound to separate RNA template strands is unlikely.

We cannot completely rule out these alternative possibilities or more complicated models to explain the role of U1 snRNP in targeting the binding of U2AF65 to the 3' splice site. However, we strongly favor the exon-bridging model described in Figure 7 because it is consistent with all of the data shown in this paper and because it is the simplest explanation for these data.

Contact B: interaction between U1 snRNP and U2AF65?

In this paper we have shown that U1 snRNP is responsible for targeting U2AF65 to the 3' splice site, but these experiments do not establish the means of contact between U1 snRNP and U2AF65. A range of possibilities include (1) direct contact involving likely protein–protein interactions, and/or RNA–protein interactions, or (2) indirect contact, which is mediated by stable complex formation with one or more additional factors in the nucleic extract. It is also possible that an additional factor is required to mediate direct contact between U1 snRNP and U2AF65.

The question of possible factors that might mediate exon bridging, contact B [Fig. 7] is indicated by the finding that purified samples of U2AF and U1 snRNP are not sufficient to reconstitute targeted p61/U2AF65 binding under a range of conditions [Fig. 6C]. Although we cannot rule out trivial explanations for this negative result, we have shown that targeted binding is reconstituted when a U2AF-depleted extract is combined with a U2AF-enriched extract. In addition, when U2AF and U1 snRNP fractions are combined with a third component, which is devoid of U2AF and U1 snRNP, targeted binding is also reconstituted. The third component was prepared by treating a crude U2AF-depleted extract with micrococcal nuclease to destroy the U1 snRNP particles in the extract. These results suggest that in addition to U2AF and U1 snRNP, an additional component(s) is required for U1 snRNP-targeted binding of p61/U2AF65 to the RNA substrate.

An additional factor might also be required for U2AF65 to discriminate the AG dinucleotide at the 3' splice site. We have found that fractions containing purified U2AF (U2AF65 + U2AF35 subunits) do not show a difference in cross-linking of p61/U2AF65 to substrates with and without an AG dinucleotide mutation at the 3' splice site, although binding is clearly reduced for substrates with polypyrimidine tract mutations (B.E. Hoffman and P.J. Grabowski, unpubl.). This is in sharp contrast to the observed discrimination of the AG dinucleotide when U2AF65 is present in the complete nuclear extract (Fig. 1A). In the complete nuclear extract, the observed reduction in p61/U2AF65 binding to an RNA substrate containing an AG dinucleotide mutation is striking because this mutation has no effect on the binding of PTB, which is present in the same binding reaction and which migrates as a protein doublet slightly faster than p61/U2AF65. Thus, these observations raise intriguing questions about how the recognition of template-bound U1 snRNP and the AG dinucleotide is achieved by U2AF65 and, at the same time, suggest assays for the identification of possible collaborating activities.

The outcome of the results shown here provides the first evidence for the involvement of a well-defined 3' splice site-binding protein, U2AF65, in U1 snRNP-facilitated exon selection. This work derives from our ongoing attempts to understand how factors and mechanisms govern the selection of alternatively spliced exons. The results we report here may be generally applicable to other genes that are alternatively spliced, such as the neural cell adhesion molecule, as selection of exon 18 is regulated by the strength of the adjacent 5' splice site (Tacke and Goridis 1991).

Our results may also bear a relationship to the regulated splicing event found in the Drosophila doublesex pre-mRNA. In this case, regulation involves 3' splice site activation by the Tra and Tra-2 proteins that bind selectively to repeated sequence elements in the adjacent female-specific exon (Hedley and Maniatis 1991; Ryner and Baker 1991). Interestingly, as with the E4 3' splice site of the preprotachykinin gene, the doublesex 3' splice site, which is subject to positive activation by Tra and Tra-2, is intrinsically poorly recognized by the constitutive splicing machinery owing to a suboptimal polypyrimidine tract. Although the cellular target of the Tra and Tra-2 proteins is currently unknown, U2AF has been suggested as one of several possible candidates to explain how exon selection is facilitated (Tian and Maniatis 1992). Perhaps both Tra/Tra-2 and U1 snRNP are capable of initiating the assembly of an activation complex that bridges part or all of the exon sequence.

The observed properties of U1 snRNP targeting of U2AF65 shown in this work may provide additional possibilities for tissue-specific regulation of alternative
splicing that are distinct from models involving direct competition of factors for binding to the 3' splice site. Alternative models may involve possible interactions of regulatory factors with U1 snRNP or U2AF65 at effector sites that block the interaction between these two splicing factors and consequently block 3' splice site selection. A prediction of these alternative models would be that a distinct class of regulatory factors will be found to have an affinity for an effector region of U1 snRNP or U2AF65, rather than an affinity for the 3' splice site.

Work in other systems points to a corresponding model of U1 snRNP-facilitated exon selection that applies to internal exons of pre-mRNAs that do not normally undergo alternative splicing. Mutations in 5' splice sites that decrease base complementarity to U1 snRNA are found to result in skipping of the exon adjacent to the mutated site (Talerico and Berget 1990). These results have led to the hypothesis that a normal characteristic of the pre-mRNA splicing process is to "define" the boundaries of each internal exon by the interaction of factors bound to the 3' and 5' splice sites of the same exon (Robberson et al. 1990). In this way, exon definition may operate normally to prevent skipping of internal constitutive exons. A general prediction of the exon definition hypothesis is that 3' exon selection would be determined by the combined strengths of the 3' and 5' splice sites flanking the exon. In agreement with this hypothesis, a recent study has shown that a subtle regulation of exon selection occurs in response to the combined strengths of 3' and 5' splice site sequence elements flanking small internal exons (Dominski and Kole 1992). However, molecular evidence for the specific factors involved in such a subtle regulation of exon selection have been lacking thus far. The communication between U1 snRNP and U2AF65 that is apparent from the work shown here may be the molecular basis by which incremental regulation of 3' splice site activity and corresponding exon selection is achieved.

**Materials and methods**

**Construction of plasmids**

Plasmids were constructed by the polymerase chain reaction (PCR) using parent plasmids RP23, RP57, and RP58 (Kuo et al. 1991). PCR-generated inserts were ligated into Bluescript vectors using restriction sites engineered into the primers (upstream, BamHI or HindIII, downstream, EcoRI), or by blunt-end ligation (HindIII). In two cases, E4CU and E4wy, the insert was constructed from two abutting PCR fragments joined by blunt end ligation. The plasmid 3'-Ad is the pRSP7 clone described previously (Konarska 1989). The plasmid 3'-E5, E4wt, and 3'-E5CU (BgIII) were treated with mung bean nuclease and recircularized with a resulting 3.3-kb fragment containing exons 4 and 5 cloned in Bluescript, Stragatene) with SpH1, then subsequently treated with exonuclease III and mung bean nuclease, followed by recircularization of the plasmid. The sequence of each clone was verified by deoxy sequence analysis. Additional details concerning plasmid construction will be made available upon request.

**Transcription and splicing reactions**

Pre-mRNA substrates were synthesized in 25-μl reactions containing 0.04 mg/ml of template DNA, T3 RNA polymerase, 0.5 mM dGTP, triphosphate, 0.4 mM each of ATP, CTP, and GTP, 0.3 mM UTP, and 2.5 μl of [α-32P]UTP (NEN 007H), as specified (Stratagene). For the differential nucleotide-labeling experiment, 2.5 μl of [α-32P]ATP, [α-32P]CTP, or [α-32P]GTP were used, and the UTP concentration was 0.4 mM. EcoRI was used as the runoff end for in vitro transcription for all plasmids, except for 3'-E4 (HaeIII) and 3'-E5, E4wt, and 3'-E5CU (BgIII). Binding reactions were carried out under splicing conditions: Each reaction, 25 μl, contained 300,000 cpm of freshly transcribed RNA substrate, 36% HeLa nuclear extract, 2 mM MgCl2, 1.5 mM ATP, and 5 mM creatine phosphate and were incubated for 20 min at 30°C.

Oligonucleotide-mediated RNase H cleavage was performed in standard splicing reactions exactly as described above, but lacking substrate RNA. To each sample, 200 ng of deoxyoligonucleotide and 1 unit of RNase H (Promega) were added, and reactions were incubated at 30°C for 45 min. Binding reactions were initiated by the addition of radiolabeled substrate RNA and subjected to UV cross-linking (see below) or native gel electrophoresis.

**UV cross-linking**

Splicing reactions were transferred to ice and irradiated immediately using a Stratalinker (Stratagene). The samples were fixed at a distance of 6.5 cm from the bulbs and routinely irradiated with 1.2 J as measured by the internal sensor. RNase A (Pharmacia) was added to a final concentration of 1 mg/ml and incubated for 20 min at 30°C. Electrophoresis sample buffer was added, and the samples were heated for 5 min at 95°C and loaded on a 12.5% SDS–PAGE (Laemml et al. 1970). The gel was fixed in 45% methanol and 9% acetic acid overnight, and dried onto Whatman paper. Cross-linked proteins were detected by autoradiography.

**Chromatography**

**U1 snRNP purification**

Nuclear extract was prepared from 8 liters of HeLa cells as described by Hinterberger et al. (1983). HeLa cells were grown in Jokick's minimal essential medium [MEM], and 5% horse serum (Sigma) to a density of 3 x 10^6 cells/ml. The extract, ~40 ml, was adjusted to 40% [wt/wt] CsCl in 20 mM Tris-HCl (pH 7.5), 15 mM MgCl2, 5 mM 2-mercaptoethanol, and 100 mM NH4Cl and centrifuged in a SW41 rotor at 40,000 rpm for 24 hr at 4°C (Brunel and Cathala 1990). The gradients were fractionated from the top into 0.65-mL volumes, and aliquots from each were assayed for the presence of snRNAs. Fractions enriched in U1 snRNP were pooled, dialyzed, and loaded onto a Protein Pak DEAE 8HR (AP-1) column (Millipore). Bound material was eluted with a linear gradient of ammonium chloride (0.1–0.4 M) U1 snRNP eluted at 275 mM, whereas snRNP containing U2, U4, U5, and U6 RNAs eluted at 345 mM. Equivalent volumes of each peak were assayed by Northern blot analysis with U snRNA probes. The fold purification of U1 snRNP (2.8 x) resulting from this two-step procedure was calculated as the ratio of the percentage of U1 snRNP recovered to the percentage of total protein recovered. The percentage of U1 snRNP recovered was estimated by Northern blot...
analysis of U1 snRNP levels in the U1 snRNP-enriched fraction in comparison with known amounts of HeLa nuclear extract using the assumption that there are 1 x 10^6 U1 snRNP particles per cell (see, e.g., Fig. 4D, lanes 3,7). U2AF purification. U2AF was purified from 8 ml of HeLa nuclear extract essentially as described, except that the initial centrifugation step was omitted (Zamore and Green 1989). A protease inhibitor cocktail [0.2 mM PMSF, 1 μM leupeptin, 0.25 μM aprotinin, 0.75 μM soybean trypsin inhibitor, and 64 μM benzamidine] was added to the extract before chromatography. The DEAE-Sephacel (10 ml) and heparin-agarose type I 15 ml columns were eluted in 2-ml fractions, and their protein content was determined by Bradford assay (Bio-Rad). The poly(U) column (5 ml) was eluted in 4-ml fractions and concentrated fivefold by spin filtration at 4°C (Millipore Ultrafree-MC). Column materials were purchased from Sigma.

Immunoblotting
The transfer and detection procedures were performed as described in Bio-Rad Bulletin 1721. Primary antibodies were used at a dilution of 1:1000. Secondary antibodies were either anti-rabbit IgG (Bio-Rad) or anti-mouse IgG (Promega) conjugated to alkaline phosphatase. NBT and BCIP (Promega) were used for color development.

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