U2 small nuclear ribonucleoprotein particle (snRNP) auxiliary factor of 65 kDa, U2AF⁶⁵, can promote U1 snRNP recruitment to 5' splice sites

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The splicing factor U2AF⁶⁵, U2 small nuclear ribonucleoprotein particle (snRNP) auxiliary factor of 65 kDa, binds to pyrimidine-rich sequences at 3' splice sites to recruit U2 snRNP to pre-mRNAs. We report that U2AF⁶⁵ can also promote the recruitment of U1 snRNP to weak 5' splice sites that are followed by uridine-rich sequences. The arginine- and serine-rich domain of U2AF⁶⁵

is critical for U1 recruitment, and we discuss the role of its RNA–RNA annealing activity in this novel function of U2AF⁶⁵.

Key words: arginine- and serine-rich domain (RS domain), psoralen, sex-lethal, splicing, U1 small nuclear ribonucleoprotein particle (snRNP), U2 auxiliary factor.

INTRODUCTION

Removal of intervening sequences (introns) from mRNA precursors (pre-mRNAs) and splicing together the coding sequences (exons) is essential to generate translatable mRNAs in eukaryotes. Regulation of splice site (ss) choice in different cells or developmental stages can generate multiple mRNA and protein isoforms, thus contributing to proteome complexity [1,2]. Exon–intron boundaries are defined by short and rather degenerate sequence motifs. Recognition of these sequences is achieved by small nuclear ribonucleoprotein particles (snRNPs) and proteins not directly associated with the snRNPs, which together constitute the spliceosome, the enzymic machine that assembles on pre-mRNAs and is responsible for intron removal [3].

U1 snRNP recognizes the 5' end of introns at early stages of spliceosome assembly. The 5' end of its RNA component, U1 small nuclear RNA (snRNA), establishes base-pairing interactions with the 5' ss region [4–6]. In addition, interactions between the pre-mRNA and U1 snRNP proteins [7–10], as well as protein–protein interactions between U1 snRNP polypeptides and proteins that associate with the pre-mRNA in the vicinity of 5' ss sequences [11–13], also contribute to U1 snRNP recruitment. One such protein, T-cell intracellular antigen-1 (TIA-1), is essential for U1 snRNP binding to weak 5' ss that are followed by uridine (U)-rich sequences [14,15]. Binding of TIA-1 to the U-rich stretch, and consequently U1 snRNP recruitment and splicing of one of these pre-mRNAs, male-specific lethal 2 (msl-2), can be inhibited *in vitro* by the *Drosophila* protein sex-lethal (SXL), a splicing regulator that also binds to U-rich sequences [16].

The intron 3' ss region is first recognized by the branch-point (BP) binding protein/splicing factor 1 and by the 65 and 35 kDa subunits of the U2 auxiliary factor (U2AF), which bind to the BP [17,18], polypyrimidine (Py)-tract [19] and 3' ss AG dinucleotide [20–22] respectively. Binding of these factors precedes association of U2 snRNP with the BP, which involves base-pair interactions between U2 snRNA and the BP sequence [23–25], as well as interactions of U2 snRNP proteins with more upstream sequences in the pre-mRNA [26].

Several mechanisms have been proposed for how U2AF⁶⁵ can promote U2 snRNP recruitment, including interaction with U2 snRNP components [27,28] and association to the DEAH helicase UAP56 U2AF associated protein of 56 kDa that may facilitate RNA–RNA rearrangements that are required for stable U2 binding [29–32]. In addition, the N-terminal arginine- and serine-rich (RS) domain of U2AF⁶⁵ has been shown to be required for U2 snRNP recruitment [19]. The RS domain contacts the BP when U2AF⁶⁵ is bound to the Py-tract [33,34] and can promote base-pair interactions [35], including those between U2 snRNA and the BP [34]. These observations led to the proposal that the RS domain contributes to U2AF⁶⁵ activity by promoting the otherwise unstable interaction between U2 snRNA and the BP, perhaps by shielding the negative charges of the approaching phosphate backbones with the positively charged arginine side chains.

One prediction of this hypothesis is that U2AF⁶⁵ could promote base-pair interactions leading to the recruitment of other snRNPs upstream from U-rich sequences. In the present paper, we show that U2AF⁶⁵ can promote U1 snRNP binding to weak 5' ss that are followed by U-rich sequences, and that the RS domain is critical for this activity.

EXPERIMENTAL

RNAs

Uniformly labelled msl-2 transcripts corresponding to part of the first exon and the 5' half of the intron were generated by *in vitro* transcription using, as a template, pGEM-msl-2 [36] digested with *Dra*III. UG1 and UG2 mutant msl-2 RNAs, with guanidine substitutions at 25 % and 50 % of the uridines downstream from the msl-2 5' ss, were described in [15], and adenovirus major late (AdML) RNAs generated as described in [37].

Glutathione S-transferase (GST) fusion proteins GST–U2AF⁶⁵, GST–U2AF⁶⁵ΔRS (U2AF⁶⁵ with deleted RS domain), GST–RS (RS domain), GST–USx (chimaeric protein between the N-terminal amino acids of U2AF⁶⁵ and SXL) and GST–U2AF⁶⁵ΔU2AF³⁵ΔUAP56 (U2AF⁶⁵ with deleted residues 95–161,

Abbreviations used: AdML, adenovirus major late; BP, branch point; GST, glutathione S-transferase; msl-2, male-specific lethal 2; Py, polypyrimidine; RRM, RNA-recognition motif; RS domain, arginine- and serine-rich domain; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; ss, splice site(s); SXL, sex-lethal; TIA-1, T-cell intracellular antigen-1; U-rich; uridine-rich; U1–70K, U1 snRNP-specific protein of 70 kDa; U2AF, U2 snRNP auxiliary factor; UAP56, U2AF associated protein of 56 kDa; USx, chimaeric protein between the N-terminal amino acids of U2AF⁶⁵ and SXL.

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including the binding sites for U2AF³⁵ and UAP56) were expressed in and purified from *Escherichia coli* [29,34,38].

Preparation of HeLa nuclear extracts and poly-U depletion

HeLa nuclear extracts were prepared according to the procedure described by Dignam et al. [39]. Chromatographic depletion of nuclear extracts on poly-U–Sepharose (Pharmacia), and elution of materials bound to the column with 2 M KCl was performed as described in [40].

Psoralen-mediated UV cross-linking

³²P-radiolabelled RNA (10 fmol) was incubated with 3.5 µl of HeLa nuclear extracts in the presence of 13 µg/ml 4'-aminomethyl-4,5',8-trimethylpsoralen in a total volume of 15 µl on ice. The samples were irradiated for 10 min with UV light at a wavelength of 365 nm in a Stratalinker oven at 4 °C. RNA was purified by digestion with proteinase K, phenol/chloroform extraction and ethanol precipitation, and analysed by electrophoresis on a 6 % denaturing polyacrylamide gel. As previously described in [15], daylight was avoided in psoralen cross-linking experiments using msl-2 RNA.

RNase H-mediated inactivation of U1 snRNP

RNase H-mediated inactivation was performed as previously described in [22]. An oligonucleotide complementary to positions 1–15 of the U1 snRNA was added to HeLa nuclear extracts and incubated in the presence of RNase H at 37 °C for 15 min and 30 °C for 30 min.

Cross-linking/immunoprecipitation

[α -³²P]UTP-labelled RNAs (20 fmol) were added to 2 µl of HeLa nuclear extracts and incubated in a final volume of 15 µl, complemented with buffer D [20 mM Hepes, pH 8.0, 20 % (v/v) glycerol, 0.2 mM EDTA, 100 mM KCl], for 15 min at 0 °C. The samples were irradiated with 0.6 J of UV light at a wavelength of 254 nm (Stratalinker) at 0 °C. After digestion with 1 mg/ml RNase A for 30 min at 37 °C, anti-U2AF⁶⁵ or control antibody was added and samples were incubated for 1 h at 0 °C. Anti-mouse agarose beads (10 µl; Sigma) were added and incubated for 2 h on a rotating wheel at 4 °C. The precipitates were washed with 500 mM NaCl, resuspended and fractionated by electrophoresis on SDS/polyacrylamide gels, which were then dried and exposed to film.

RESULTS

Binding of U2AF⁶⁵ to the msl-2 5' ss region

Previous *in vitro* results indicated that the human protein TIA-1 can promote binding of U1 snRNP to a 5' ss of the *Drosophila* msl-2 [15]. TIA-1 was found to recognize the U-rich stretch immediately downstream of this 5' ss. Chromatographic depletion of TIA-1 from nuclear extracts using poly-U–Sepharose columns efficiently compromised U1 snRNP binding to this 5' ss, but not to stronger 5' ss that were not followed by U-rich sequences.

Addition of the 2 M guanidine/HCl eluate of the poly-U–Sepharose column to the depleted extracts restored their activity. UV cross-linking analysis identified two proteins present in the 2 M guanidine fraction that interact with an RNA corresponding to

the msl-2 5' ss region. An approx. 42 kDa species corresponds to TIA-1 [15]. In addition, cross-linking of an approx. 65 kDa polypeptide was detected [15]. Given the affinity of the splicing factor U2AF⁶⁵ for U-rich sequences [19,41], and that its chromatographic behaviour in poly-U–Sepharose columns is similar to that of TIA-1, the 65 kDa cross-link could correspond to U2AF⁶⁵.

Immunoprecipitation using an anti-U2AF⁶⁵ monoclonal antibody [42] confirmed that the protein cross-linked to the msl-2 5' ss region was indeed U2AF⁶⁵ (Figure 1A, lane 2). Cross-linking to the 5' ss region of msl-2 was less efficient than to the 3' ss region of the same transcript, which contains a long Py-tract of 16 uridines (compare lanes 2 and 6 of Figure 1A). Cross-linking was nevertheless specific because (i) it was not detected when the 5' ss region of an adenovirus pre-mRNA was used, a substrate that does not contain a U-rich stretch downstream from the 5' ss (Figure 1A, lane 4), and (ii) cross-linking was reduced upon progressive replacement of the uridines downstream from the msl-2 5' ss by guanidines (UG1 and UG2 RNAs [15]; Figures 1B and 1C).

Taken together, these results indicate that U2AF⁶⁵ specifically recognizes U-rich sequences located downstream of the msl-2 5' ss, and mutation of these sequences compromises U2AF⁶⁵ binding.

U2AF⁶⁵ can promote U1 snRNP recruitment

To test whether U2AF⁶⁵ binding to the msl-2 5' ss region could influence U1 snRNP recruitment, psoralen-mediated cross-linking assays were carried out. Psoralen intercalates between base-paired nucleotides and, upon irradiation with long-wave UV light, covalent bonds are formed between the base-paired strands. This method has been used to detect base-pairing interactions between snRNAs and splicing signals in the pre-mRNA [43,44]. Figure 2(A) shows that a psoralen-mediated cross-link was detected upon incubation of radiolabelled msl-2 5' ss RNA with nuclear extracts (Figure 2A, lane 3), but not with extracts in which the 5' end of U1 snRNA was degraded by RNase H-mediated digestion in the presence of a complementary oligonucleotide (Figure 2A, lane 4). These results indicate that the identified cross-link corresponds to base-pairing between the 5' ss of msl-2 and U1 snRNA, a result consistent with previous reports [15,16]. The msl-2–U1 cross-link was reduced in poly-U–Sepharose-depleted nuclear extracts (Figure 2B, compare lanes 2 and 3; see also [15]). Remarkably, the msl-2–U1 cross-link was stimulated by the addition of recombinant purified GST–U2AF⁶⁵ to the extracts (Figure 2B, compare lanes 3 and 4), indicating that U2AF⁶⁵ can indeed promote U1 snRNP recruitment to the msl-2 5' ss. Consistent with this, addition of recombinant U2AF⁶⁵ (or a chromatographic fraction from HeLa nuclear extracts containing the U2AF^{65/35} heterodimer) to limited concentrations of undepleted extracts can enhance U1 snRNA cross-linking (Figure 2C, compare lanes 1 with 2 and 3).

Two lines of evidence indicate that the effects observed are specific to 5' ss followed by U-rich sequences. First, U1 snRNA cross-linking to mutant UG1 and UG2 msl-2 RNAs was very weak or undetectable and was not enhanced by an excess of U2AF⁶⁵ (Figure 2D). Secondly, cross-linking of an adenovirus pre-mRNA that contained a strong 5' ss and lacked downstream U-rich stretches was not significantly affected by depletion of poly-U binding proteins (Figure 2E, compare lanes 2 and 4), nor was it stimulated by the addition of recombinant U2AF⁶⁵ or partially purified U2AF (Figure 2F).

Figure 1 Cross-linking of U2AF⁶⁵ to the msl-2 5' ss region

(A) Cross-linking to msl-2 and AdML sequences. Radiolabelled msl-2 or AdML RNAs corresponding to part of exon 1 and the 5' half of the intron (indicated by a box and a line respectively) were incubated with the 2 M guanidine/HCl eluate of a poly-U-Sepharose column loaded with HeLa nuclear extracts at 1 M KCl and subsequently washed with 2.5 M KCl. After irradiation with UV light at a wavelength of 254 nm and digestion with RNase A, immunoprecipitation with antibodies against U2AF⁶⁵ (αU2AF) or a control antibody (αMyc) was carried out. Precipitates were washed, the proteins were fractionated by SDS/PAGE and exposed to film. The position of U2AF⁶⁵ is indicated. (B) Sequence of the 5' ss regions of the RNAs used in this study. (C) Reduced U2AF⁶⁵ cross-linking to msl-2 mutated at the U-rich stretch downstream from the 5' ss. Cross-linking/immunoprecipitation was carried out as in (A) using msl-2 UG1 and UG2 mutant RNAs, which contain guanine substitutions at 25 % and 50 % of the uridines downstream from the msl-2 5' ss respectively.

We conclude that U2AF⁶⁵ can promote U1 snRNP recruitment to 5' ss, depending upon the presence of U-rich sequences downstream of the ss.

U2AF⁶⁵ RS domain is essential for U1 snRNP recruitment

To analyse the domain requirements for this function of U2AF⁶⁵, the activity of a series of deletion mutants (expressed as fusion proteins with GST) was tested in poly-U-Sepharose-depleted extracts. Although full-length U2AF⁶⁵ partially restored the activity of the extracts (Figure 3, lane 3), a mutant lacking the N-terminal RS domain was inactive in these assays (Figure 3, lane 4). The RS domain by itself, however, was also unable to stimulate U1 cross-linking (Figure 3, lane 5). The results indicate that both the RS and the RNA-recognition motif (RRM) domains are required for the activity of U2AF⁶⁵ in U1 snRNP recruitment.

Interaction of U2AF⁶⁵ with other splicing factors, the 35 kDa subunit of U2AF (U2AF³⁵) and the DEAH-box-containing factor UAP56, can influence U2AF activity in U2 snRNP recruitment [29,45–48]. Interaction with these factors seems dispensable for the U1 snRNP recruitment activity of U2AF⁶⁵, because a mutant in which the region of interaction with these factors was deleted was fully functional in the complementation assays (Figure 3, lane 6).

It has been shown that the protein SXL binds to the U-rich sequence downstream of the msl-2 5' ss and prevents U1 snRNP recruitment [16]. USx, a chimaeric protein between the N-terminal amino acids of U2AF⁶⁵ and SXL, displays U2AF activity with the RNA-binding specificity of SXL, both *in vitro* and *in vivo* [38,49]. Remarkably, this USx fusion protein was a potent activator of U1 snRNP binding in complementation of poly-U-depleted extracts (Figure 2B, compare lanes 3 and 5). This result suggests that the N-terminal RS domain-containing region of U2AF⁶⁵ is sufficient to convert a repressor of U1 snRNP binding into an activator of U1 snRNP recruitment.

DISCUSSION

The present paper describes the unexpected observation that U2AF⁶⁵, extensively characterized as an activity factor that is necessary for the recruitment of the spliceosomal U2 snRNP to 3' ss, can also promote recognition of a subset of 5' ss by U1 snRNP. These observations reveal a novel activity for U2AF⁶⁵ and demonstrate that it can promote the recruitment of snRNP complexes different from U2 snRNP. Analysis of the domain requirements in U2AF⁶⁵ lends support to the proposal that U2AF⁶⁵ promotes snRNP recruitment, at least in part, by using its RS domain to promote RNA base-pair interactions. For this activity, it is essential that U2AF⁶⁵ binds to U-rich sequences that serve as binding sites for the protein on the pre-mRNA and allow appropriate positioning of the RS domain. The results underscore the flexibility of the splicing machinery to facilitate recognition of a variety of ss sequences, a finding with implications for alternative splicing regulation.

Previous work implicated the RNA-binding protein TIA-1 in promoting the recruitment of U1 snRNP to weak 5' ss [14,15]. The results of the present study show that a second poly-U binding protein, U2AF⁶⁵, promotes U1 snRNP to the msl-2 5' ss, which is characterized by a relatively weak 5' ss followed by U-rich sequences. It has been difficult to measure the relative contributions of the two proteins in 5' ss recognition in the context of the complete splicing process, because U2AF⁶⁵ could also stimulate 5' ss definition indirectly from the 3' ss, through bridging interactions across the intron.

Recent results indicate that TIA-1 contacts the U1 snRNP-specific protein U1C, and that this interaction has an important role in snRNP recruitment by TIA-1 [13]. Indeed, protein–protein interactions between splicing-regulatory factors and U1 snRNP-specific components are a recurrent theme in the regulation of

Figure 2 U2AF⁶⁵ can assist U1 snRNP recruitment to msl-2 5' ss

(A) Psoralen-mediated cross-linking between the msl-2 5' ss and U1 snRNA. Radiolabelled msl-2 RNA was incubated with HeLa nuclear extracts (NE) in the presence (+) or absence (−) of psoralen. After irradiation with UV light of a wavelength of 365 nm, the RNA was purified and fractionated on denaturing polyacrylamide gels. NE U1 k.o. indicates nuclear extracts in which the 5' end of U1 snRNA was degraded by RNase H. The positions of msl-2 RNA and products of cross-linking are indicated. (B) Psoralen-mediated cross-linking in poly-U-depleted extracts. Assays were performed as in (A), using either standard nuclear extracts (NE) or poly-U depleted nuclear extracts (NEΔpolyU), in the presence or absence of 40 ng/μl recombinant purified U2AF⁶⁵ or a chimaeric protein containing the RS domain of U2AF⁶⁵ fused to the RNA-binding domains of SXL (USx). (C) U1 snRNA cross-linking can be enhanced by addition to nuclear extracts of recombinant U2AF⁶⁵ or a chromatographic fraction containing the U2AF^{65/35} heterodimer (2 M KCl eluate of the poly-U-Sepharose used for chromatographic depletion of U2AF). Assays were performed as in (A) using limiting amounts of nuclear extract. (D) Diminished U1 snRNA cross-linking to msl-2 UG1 and UG2 mutant RNAs. Assays were carried out as in (A) using the indicated RNAs, whose sequences are shown in Figure 1(B). (E) U1 snRNA cross-linking to AdML RNA is not affected by depletion of nuclear extracts on poly-U columns. Psoralen-mediated assays were performed as in (B) using radiolabelled RNA corresponding to the first exon and the 5' half of the intron of an AdML promoter transcript. (F) Neither recombinant U2AF⁶⁵ nor a chromatographic fraction containing U2AF stimulate U1 snRNA cross-linking to AdML RNA. Assays were carried out as in (E).

Figure 3 Domains of U2AF⁶⁵ required for U1 snRNP recruitment

Complementation of poly-U-depleted nuclear extracts (NEΔpolyU) by recombinant U2AF⁶⁵ and mutant derivatives was carried out as in Figure 2(B). Only the products of U1 snRNA–msl-2 5' ss cross-linking are shown. ΔRS corresponds to deletion of residues 1–63 of U2AF⁶⁵; RS corresponds to residues 1–63 of U2AF⁶⁵, which are rich in arginine–serine dipeptides; ΔU2AF³⁵ΔUAP56 corresponds to deletion of residues 95–161 of U2AF⁶⁵, which includes the binding sites for the splicing factors U2AF³⁵ and UAP56. All recombinant proteins were purified as fusion proteins with GST.

5' ss recognition. For example, the *Drosophila* protein P-element somatic inhibitor ('PSI'), which is part of a regulatory complex that is important for the retention of the P-element third intron in somatic cells, contacts the U1 snRNP-specific protein of 70 kDa (U1–70K) and helps to recruit the snRNP onto a cryptic 5' ss, thus preventing activation of the bona fide 5' ss and therefore inhibiting splicing of the intron [12,50,51]. U1–70K also seems to be the U1 component that is contacted by the serine/arginine-rich protein alternative splicing factor/splicing factor 2 ('AFS/SF2') to promote 5' ss utilization [11]. The RS domains of both proteins are involved in protein–protein interactions [52,53].

The results shown in Figures 2 and 3 indicate that the N-terminal RS domain, either in combination with the RRM of U2AF⁶⁵ or with those of SXL, is important for the activity of the protein in U1 snRNP recruitment. No protein–protein interactions, however, have been detected between the RS domain of U2AF⁶⁵ and those of other splicing factors [52].

An alternative function has been proposed for this domain in U2 snRNP recruitment, based on three lines of experiments. First, the RS domain, essential for U2 binding [19], could be replaced by other RS domains or even by a synthetic domain composed of seven RS dipeptides. Remarkably, activity was maintained upon the substitution of lysine by arginine, but not by alanine. Serine could be replaced by a variety of amino acids, with the exception of negatively charged residues [34]. These observations suggested that the only structural determinant that is relevant for the function of the RS domain *in vitro* is the presence of a positively charged surface at the N-terminus of U2AF⁶⁵. A second line of experiments showed that the RS domain of U2AF⁶⁵ contacts the BP sequence upstream from the Py-tract when the Py-tract is bound by U2AF⁶⁵ RRMs [33,34]. The third line of experimentation showed that the U2AF⁶⁵ RS domain promotes annealing of nucleic acids [35], and that U2AF⁶⁵ can promote base pairing between *in vitro* transcribed U2 snRNA and the BP sequence [34]. Based on the correlation between the ability of RS domain mutants to (i) promote splicing, (ii) contact the BP and (iii) promote U2 snRNA–BP base-pair interactions, it was proposed that the function of the U2AF⁶⁵ RS domain is to promote the targeted annealing of otherwise unstable base-pair interactions between nucleic acids.

In addition to promoting U2 snRNA–BP interactions, it is conceivable that U2AF⁶⁵ could be involved in promoting other RNA–RNA interactions, including U1 snRNA–5' ss interactions, depending upon the presence of U-rich sequences downstream from the region of base-pairing. Consistent with this hypothesis, base pairing between U2 and U6 snRNAs was found to be

decreased in extracts depleted chromatographically of U2AF, and partially restored upon addition of recombinant U2AF⁶⁵ [34]. Interestingly, a uridine stretch follows the region of base pairing in U6 snRNA [54].

The molecular basis for the annealing function of the RS domain may be related to the shielding of negative charges associated with the phosphodiester backbones of the approaching strands. A similar mechanism has been proposed for the stabilizing effect that the positively charged tails of some Sm core proteins have on U1 binding [32]. It is likely that multiple interactions stabilize the functional association of U1 snRNP with 5' ss [7]. This can explain why the snRNP can be recruited even in the absence of base-pair interactions between U1 snRNA and the 5' ss [55]. Regulation, however, can be achieved by the binding of a single protein (SXL) to a critical sequence bound by at least two auxiliary factors (TIA-1 and U2AF⁶⁵).

Conversely, a single factor may provide more than one activity. We cannot rule out, for example, that protein–protein interactions that are mediated by the RS domain or other regions of U2AF⁶⁵ do not provide additional means by which the factor can promote snRNP recruitment. Similarly, our results illustrate how a splicing factor can provide various distinct activities in spliceosome assembly depending on the sequence configuration of the ss regions, an observation relevant to the regulation of alternative pre-mRNA splicing.

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