Distinct Factor Requirements for Exonic Splicing Enhancer Function and Binding of U2AF to the Polypyrimidine Tract*

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Exonic splicing enhancer (ESE) sequences are important for the recognition of adjacent splice sites in pre-mRNA and for the regulation of splice site selection. It has been proposed that ESEs function by associating with one or more serine/arginine-repeat (SR) proteins which stabilize the binding of the U2 small nuclear ribonucleoprotein particle (snRNP) auxiliary factor (U2AF) to the polypyrimidine tract upstream of the 3' splice site. We have tested this model by analyzing the composition of splicing complexes assembled on an ESE-dependent pre-mRNA derived from the *doublesex* gene of *Drosophila*. Several SR proteins and hTra2β, a human homolog of the *Drosophila* alternative splicing regulator Transformer-2, associate with this pre-mRNA in the presence, but not in the absence, of a purine-rich ESE. By contrast, the 65-kDa subunit of U2AF (U2AF-65 kDa) bound equally to the pre-mRNA in the presence and absence of the ESE. Time course experiments revealed differences in the levels and kinetics of association of individual SR proteins with the ESE-containing pre-mRNA, whereas U2AF-65 kDa bound prior to most SR proteins and hTra2β and its level of binding did not change significantly during the course of the splicing reaction. Binding of U2AF-65 kDa to the ESE-dependent pre-mRNA was, however, dependent on U1 snRNP. The results indicate that an ESE promotes spliceosome formation through interactions that are distinct from those required for the binding of U2AF-65 kDa to the polypyrimidine tract.

Pre-mRNA splicing requires the assembly of a large multi-subunit complex, the spliceosome, which consists of four small nuclear ribonucleoprotein particles (U1, U2 U4/U6, and U5 snRNPs) and numerous non-snRNP protein factors (1, 2). Biochemical and genetic studies have provided evidence that over 50 proteins are required for constitutive splicing, many of which are highly conserved between yeast and mammals. In metazoans, a large number of non-snRNP protein splicing factors have been identified which contain domains rich in alternating serine and arginine residues (RS domains) (3–6). A subgroup of these proteins, the SR family, contain one or two N-terminal RNA recognition motifs and a phosphorylated C-terminal RS domain. SR family proteins include SRp20, ASF/ SF2, SC35, 9G8, SRp30c, SRp40/HRS, SRp55, SRp75, and a more distantly related protein, p54. These proteins promote general splicing activity and also function in regulated pre-mRNA splicing. SR proteins interact with each other and also with snRNP-associated proteins that contain RS domains and these interactions are mediated by phosphorylated RS domains (7–12). These protein-protein interactions are important for promoting the recognition and pairing of splice sites and for subsequent steps in spliceosome assembly.

The assembly of spliceosomes involves an ordered association of snRNPs and non-snRNP protein factors with the pre-mRNA (1). At an early stage in assembly, U1 snRNP binds to sequences at the 5' splice site and promotes the binding of U2 snRNP to the intron branch site (13, 14). The stable binding of U2 snRNP to the branch site also requires the U2 auxiliary factor (U2AF), which consists of two subunits, U2AF-65 kDa and U2AF-35 kDa (15). U2AF-65 kDa contains a short N-terminal RS domain followed by three RRMs and U2AF-35 kDa contains a short C-terminal RS domain (16, 17). U2AF binds through the 65-kDa subunit to the polypyrimidine tract located between the branch site and 3' splice site (18). Following the assembly of a U2 snRNP-containing pre-splicing complex, U4/U6 and U5 snRNPs enter the assembly pathway as a pre-formed tri-snRNP particle which, together with additional protein factors, form an assembled spliceosome.

In higher eukaryotes, the sequences specifying splice sites and the branch site are weakly conserved and are often surrounded by many cryptic splice sites that are not selected. Moreover, many pre-mRNAs in metazoan cells contain alternative splice sites that must be recognized in a regulated fashion to generate specific mRNA variants (reviewed in Refs. 19 and 20). Additional sequence elements within a pre-mRNA are important for splice site recognition and one type of element is the exonic splicing enhancer (ESE). One or more SR proteins bind to ESEs and promote the recognition of adjacent splice sites (reviewed in Refs. 3–5 and 21). ESEs are often purine-rich although recent studies employing SELEX procedures have demonstrated that diverse sequences can function as enhancers in conjunction with different SR proteins (22, 23). Recently, it was demonstrated that the splicing coactivator SmRm160/300 (complex of SR-related matrix proteins of 160kDa and 300kDa) is required for ESE-dependent splicing (24, 25). The association of SmRm160/300 with an ESE-dependent pre-mRNA requires U1 snRNP, factors bound to the ESE, and is stabilized by U2 snRNP. Independently of pre-mRNA, SmRm160/300 interacts specifically with U2 snRNP and hTra2β, a human homolog of the *Drosophila* alternative splicing regulator Transformer-2 that binds to purine-rich ESEs (26). It was proposed that SmRm160/300 functions in ESE-dependent splicing by mediating critical interactions between ESE-bound components and the snRNP machinery of the spliceosome. However, the mech-
EXPERIMENTAL PROCEDURES

Antibodies—Antibodies used in this study are the murine monoclonal mAb104 (29), and the rabbit polyclonal anti-U2AF-65 kDa (30) and anti-hTra2β (26).

Nuclear Extracts—HeLa nuclear extracts were prepared essentially as described by Dignam et al. (31). Nuclear extracts depleted of U1 snRNP or U2 snRNP were prepared as described by Blencowe and Lamond (32) and are identical to the preparations described in Blencowe et al. (24).

Splicing Complex Selection Assay—Affinity selection of splicing complexes was performed using conditions modified from those described by Ryder et al. (33) for the analysis of the snRNPs composition of splicing complexes. Selections were performed from 225-μl splicing reactions (see below) containing near-saturation levels of the biotinylated dsx pre-mRNAs. After affinity selection of splicing complexes with streptavidin-agarose beads (Sigma), the beads were washed three times (5 min/wash, rotating at 4 °C) with a 300 mM KCl wash buffer (WB300): 10 mM Tris, pH 7.6, 300 mM KCl, 2.5 mM MgCl2, 5 mM potassium fluoride, 5 mM β-glycerophosphate). Proteins associated with the biotinylated dsx pre-mRNAs were eluted from the beads with 400 μl of a high salt buffer (65 mM KCl, 2M NaCl, 20 mM EDTA, 5 mM potassium fluoride, 5 mM β-glycerophosphate), and precipitated with 0.25 volumes of 100% trichloroacetic acid containing 4 mg/ml deoxycholate. The protein pellets were washed with acetone and resuspended in SDS sample buffer for immunoblot analysis.

In Vitro Transcription of Pre-mRNA Substrates—[32P]UTP-labeled and unlabeled dsx pre-mRNAs (34) were transcribed in vitro in the presence of biotin-11-UTP (Sigma), using T7 RNA polymerase (Amerham Pharmacia Biotech) as per the manufacturer’s instructions. The final concentrations of ribonucleotides in the transcription reactions were exactly as described in Eldridge et al. (25).

Splicing Assays—Splicing reactions were carried out essentially as described by Eldridge et al. (25). The splicing reaction in Fig. 1A was analyzed on a 15% denaturing polyacrylamide gel.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis—SDS–polyacrylamide gel electrophoresis and immunoblots were performed essentially as described by Harlow and Lane (35). The immunoblots were developed using secondary antibodies conjugated to horseradish peroxidase and chemiluminescence detection (NEN Life Science Products Inc.), as per the manufacturer’s instructions.

RESULTS

ESE-dependent Association of SR Family Proteins and hTra2β with a dsx Pre-mRNA—We have investigated the protein composition of splicing complexes assembled on an ESE-dependent substrate derived from exons 3 and 4 of the Drosophila doublesex (dsx) gene, which contains a suboptimal polypyrimidine tract and a typical mammalian ESE consisting of GAA repeats in the 3′ exon (34). This pre-mRNA is spliced with increased efficiency as the number of GAA repeats in the ESE is increased (25, 34). Biotinylated derivatives of the dsx pre-mRNA were synthesized that lack an ESE (dsxΔE+bio), that contain an ESE with either three (dsx(GAA)3bio), or six (dsx(GAA)6bio) GAA repeats. These biotinylated pre-mRNAs are spliced with similar efficiency as the unmodified substrates (Fig. 1A and data not shown). Splicing complexes assembled on the biotinylated pre-mRNAs were affinity-selected on streptavidin-agarose beads and analyzed by immunoblotting with mAb104, which detects a phosphoepitope within the RS domains of many SR proteins (29, 49) (Fig. 1B).

The GAA repeat ESE promoted the association of several SR-related proteins with the dsx pre-mRNA, including species migrating at 30, 40, 55, and 75 kDa. Low levels of these proteins were detected in complexes on the dsxΔE pre-mRNA (Fig. 1, lane 5). These levels increased significantly on the dsx(GAA)3 pre-mRNA (lane 6). A further increase was detected for the 40-, 55-, and 75-kDa proteins on the dsx(GAA)6 pre-mRNA (lane 7). Of the different proteins detected by mAb104, the 40-kDa species was the most strongly enriched in response to the increase in number of GAA repeats. The amount of each mAb104-reactive protein that associates with the dsx(GAA)6 pre-mRNA, depending on the species, is approximately 1–3% of the total amount of the protein in the splicing reaction. The selection of the different SR proteins on the dsx pre-mRNAs was not due to nonspecific interactions with the streptavidin-agarose beads since none of the proteins were detected in selections from reactions incubated without pre-mRNA (lane 3), or with a dsx(GAA)6 pre-mRNA lacking biotin residues (lane 4). Moreover, only a specific subset of SR-related proteins was selected since at least one prominent protein detected by mAb104 in total extract, migrating at ~130 kDa, was not enriched on the dsx(GAA)6 pre-mRNA (compare lanes 1 and 7).

Complexes affinity selected on the different dsx pre-mRNAs were also immunoblotted with antibodies specific for SRm160 and SRm300. Previous immunoprecipitation experiments performed under low salt (100 mM NaCl) conditions demonstrated that increased levels of these proteins associate with the dsx pre-mRNA as the number of GAA repeats in the ESE increase (25). However, it was found that they dissociate under the higher salt conditions (300 mM KCl) used in the affinity selection assays in the present study (data not shown). This indicates that SRm160/300 and possibly additional SR-related proteins are more weakly associated with the dsx pre-mRNA than the SR proteins detected by mAb104.

The 30-, 55-, and 75-kDa proteins detected by mAb104 most likely correspond to the defined SR family proteins of these sizes. Candidates for the 40-kDa species include the SR family protein SRp40/HSR (36, 37) and hTra2α and or hTra2β. The latter two proteins are human homologs of the Drosophila alternative splicing factor Transformer-2 (38, 39), which are detected by mAb104 and bind specifically to ESEs containing GAA repeats (26). Consistent with the 40-kDa species containing one of the hTra2 proteins, a polyclonal antibody specific for hTra2β (26) detected increased levels of this protein in the dsx splicing complexes as the number of GAA repeats increase (Fig. 1C). The level of enrichment of hTra2β was similar to that observed for the 40-kDa species detected by mAb104, indicating that a significant fraction of the latter may correspond to hTra2β (compare Fig. 1, B and C). The results in Fig. 1 provide evidence that a GAA repeat ESE promotes the association of a specific set of SR family proteins and hTra2β with the dsx pre-mRNA.

Binding of U2AF-65 kDa to the dsx Pre-mRNA Does Not Require an ESE—In previous studies, it was proposed that ESEs function by promoting the binding of U2AF-65 kDa to adjacent, suboptimal, polypyrimidine tracts (27, 40). To determine whether this is the case for the dsx pre-mRNA in the present study, protein samples from the affinity selections in Fig. 1B were probed with an antibody specific for U2AF-65 kDa (Fig. 2A). In contrast to the ESE-dependent association of SR proteins and hTra2β with the dsx pre-mRNA, U2AF-65 kDa bound to this substrate in the absence of the ESE (lane 3). Moreover, its level of binding did not change significantly in the presence of the ESE (compare lanes 3 and 5). The slight in-
increase in the level of U2AF-65 kDa detected in the selection with the dsx(GAA)₆ pre-mRNA, compared with the levels observed in the selections with the dsx3E and dsx(GAA)₆ pre-mRNAs (compare lane 4 with lanes 3 and 5) was not observed in several repeat experiments and was attributed to a minor loading difference. As in the case of the SR proteins detected in the dsx splicing complexes, binding of U2AF-65 kDa to the dsx pre-mRNA was not due to a nonspecific interaction since it was not selected from reactions containing the non-biotinylated dsx(GAA)₆ pre-mRNA (lane 2), nor was it selected from a reaction containing a biotinylated 5'-half pre-mRNA lacking a polypyrimidine tract (Fig. 2B, compare lanes 2 and 3). The results indicate that binding of U2AF-65 kDa to the dsx pre-mRNA is not dependent on the ESE.

**Kinetics of Association of SR Proteins and U2AF-65 kDa with the dsx Pre-mRNA**—To extend the above observations, we next analyzed the kinetics of association of SR proteins and U2AF-65 kDa with the dsx(GAA)₆ pre-mRNA. Complexes assembled on this substrate were affinity selected after different incubation times in a splicing reaction. Proteins recovered from the splicing complexes were immunoblotted with mAb104 (Fig. 3A) and the anti-U2AF-65 kDa antibody (Fig. 3C). Interestingly, different SR proteins were detected by mAb104 on the pre-mRNA at different times during the reaction. “SRp75” bound on ice, and its level did not change significantly during incubation of the splicing reaction at 30 °C (compare lanes 3–5). By contrast, the other SR proteins and hTra2β were detected at very low levels on ice and increased rapidly during the incubation at 30 °C, reaching essentially maximal levels during the first 10 min of the reaction (compare lanes 3–5; data not shown). Consistent with an energy requirement for the association of these SR proteins with the pre-mRNA, they bound at significantly lower levels in splicing reactions incubated without ATP (data not shown). The levels of the SR proteins selected at 10 min in the presence of ATP (lane 5) remained constant for a further ~50 min, after which they declined (data not shown). As observed in Fig. 1B, the 40-kDa species detected by mAb104, corresponding in part to hTra2β, was enriched to a higher extent than the other SR proteins on the dsx(GAA)₆ pre-mRNA. An analysis of the supernatant fractions from the selections by immunoblotting with mAb104 did not reveal significant differences in the levels of the non-selected proteins, which comprise at least 97% of the total amounts of these proteins in the splicing reaction (Fig. 3B, compare lanes 1–3). This indicates that the increased levels of detection of the SR proteins on the dsx(GAA)₆ pre-mRNA is not due to changes in the levels and/or phosphoepitope status of the SR proteins prior to their association with the pre-mRNA. However, a slower migrating 30-kDa species was enriched in splicing complexes selected at 10 min compared to complexes selected at 0 and 5 min (Fig. 3A, compare lanes 1–3). It is possible that this protein represents a distinct 30-kDa species, or else a modification to an existing protein within the affinity selected splicing complexes (see “Discussion” below). The results in Fig. 3A indicate that the ESE-dependent recruitment of several SR family proteins and hTra2β on the dsx(GAA)₆ pre-mRNA occurs during the first 10 min of a splicing reaction. Moreover, these proteins differ in the levels and kinetics with which they associate with the dsx pre-mRNA.

The complexes affinity-selected in Fig. 3A were next immu-
FIG. 2. U2AF-65 kDa binds to the dsx pre-mRNA equally in the presence and absence of the ESE. A, splicing complexes affinity selected as in Fig. 1B were analyzed by immunoblotting with a polyclonal antibody specific for U2AF-65 kDa (30). B, complexes selected from splicing reactions containing either the dsx(GAA)₆ pre-mRNA (lane 2) or a control biotinylated RNA consisting of a 5′-half pre-mRNA substrate lacking a polypyrimidine tract (lane 3, see “Experimental Procedures”). The samples were separated on a 12% SDS-polyacrylamide gel and immunoblotted with the anti-U2AF-65 kDa antibody. The amount of nuclear extract loaded in lane 1, and the amount used in each affinity-selection in lanes 2 and 3, is as described in the legend to Fig. 1B.

Binding of U2AF-65 kDa to the dsx Pre-mRNA Requires U1 snRNP—We next investigated the factor requirements for binding of U2AF-65 kDa to the dsx substrate. It has been recently reported that U1 snRNP is required for the binding of U2 snRNP to the dsx(GAA)₆ pre-mRNA used in the present study (25). It has also been reported that U1 snRNP can promote the cross-linking of U2AF-65 kDa to an upstream polypyrimidine tract across an exon and also to the polypyrimidine tract of a constitutively spliced pre-mRNA containing a single intron (41, 42). We therefore investigated if it is also required for the binding of U2AF-65 kDa within the context of cross-intron interactions during ESE-dependent splicing on the biotinylated dsx(GAA)₆ pre-mRNA. Splicing complexes assembled on this substrate were affinity selected from splicing reactions depleted of individual snRNPs and then immunoblotted with the anti-U2AF-65 kDa antibody (Fig. 4). Depletion of U1 snRNP resulted in a significant reduction in the level of U2AF-65 kDa binding to the dsx(GAA)₆ pre-mRNA compared with its level of binding in a “mock”-depleted extract (compare lanes 2 and 4). This reduction was not due to a nonspecific loss since depletion of U2 snRNP did not reduce the level of U2AF-65 kDa binding (compare lanes 2 and 5), and mixing equal amounts of the U1 and U2 snRNP-depleted extracts restored binding to the level observed in the mock-depleted reaction (compare lanes 2 and 6). These results indicate that U1 snRNP functions in stabilizing the binding of U2AF-65 kDa to the dsx(GAA)₆ pre-mRNA.

DISCUSSION

The results in the present study provide evidence that a purine-rich ESE is important for the stable association of hTra2β and several SR family proteins with the dsx pre-mRNA, but not U2AF-65 kDa. Instead, binding of U2AF-65 kDa is promoted by U1 snRNP. This indicates that an ESE promotes the formation of splicing complexes through interac-
tions that are separate from those required for the binding of U2AF-65 kDa to the polypyrimidine tract. These findings are in contrast to those of previous studies in which it was reported that cross-linking of U2AF-65 kDa to pre-mRNAs containing suboptimal polypyrimidine tracts is increased in the presence of an ESE (27, 43, 44). However, in these studies the level of U2AF-65 kDa cross-linking was not found to correlate with increased splicing activity promoted by the ESE. In another study employing recombinant factors in the absence of nuclear extract, it was observed that efficient cross-linking of U2AF-65 kDa to the dsx pre-mRNA required the addition of a single SR family protein and U2AF-35 kDa (28). In the present study, binding of U2AF-65 kDa to the dsx pre-mRNAs was assayed in splicing reactions containing total nuclear extract without added factors. It is possible that the experimentally defined levels of SR proteins and U2AF subunits in the previous study resulted in the promotion of interactions that do not normally occur in splicing reactions. The present findings are consistent with a very recent report demonstrating that cross-linking of U2AF-65 kDa to two different ESE-dependent pre-mRNAs occurs with equal efficiency in the presence or absence of an ESE, and that immunodepletion of U2AF-35 kDa does not prevent ESE-dependent splicing in vitro (45). Moreover, it has also been reported recently that the RS domain of the Drosophila homolog of U2AF-35 kDa, which was proposed to mediate interactions with ESE-bound SR proteins (7), is dispensable for viability and also the regulation of dsx pre-mRNA splicing (46).

An ESE consisting of six GAA repeats was sufficient for promoting the association of several SR proteins and hTra2β with the dsx pre-mRNA. This is consistent with previous studies demonstrating that SR proteins of these sizes can associate specifically with short RNAs consisting only of GAA repeats (23, 34). Interestingly, in the present study these proteins were detected on the dsx(GAA)₆ pre-mRNA at different times during the splicing reaction, with the stable binding of “SRp75” appearing earlier than the 55-kDa, 40-kDa, hTra2β, and 30-kDa SR protein(s). This indicates that different SR proteins and hTra2β may associate with the dsx pre-mRNA individually rather than as a pre-assembled complex. The increased detection over time of a slower migrating 30-kDa protein(s) detected by mAb104 on the dsx(GAA)₆ pre-mRNA could reflect the recruitment of a distinct 30-kDa SR protein, or else the modification of one or more 30-kDa SR protein(s) already bound to this substrate. The latter case would be consistent with the previous observation that phosphorylation of ASF/SF2 is important for ESE-dependent splicing (12). The results in the present study demonstrate that the SR protein composition of complexes formed on the dsx pre-mRNA undergoes dynamic changes during ESE-dependent splicing.

We have demonstrated recently that the level of binding of U1 snRNP to the dsx pre-mRNA substrate in the present study is approximately equivalent in the presence and absence of the ESE (25). It was therefore unexpected that only minor levels of SR family proteins and hTra2β were detected on the dsx pre-mRNA in the absence of the ESE, since previous reports have indicated that SR proteins such as ASF/SF2 promote the binding to U1 snRNP to the 5’ splice site (8, 12). However, as in the previous U2AF binding studies cited above (28), these earlier studies assayed binding of U1 snRNP in reactions containing added purified components, whereas we have assayed binding in splicing reactions without added purified proteins. Although the low levels of SR proteins detected on the dsxΔE pre-mRNA could represent a population that functions in promoting the binding of U1 snRNP, the present results, taken together with those of Eldridge et al. (25), indicate that the increased SR protein and hTra2β association with the dsx pre-mRNA promoted by the ESE does not significantly influence U1 snRNP binding.

We also demonstrated recently that U1 snRNP is strongly required for the association of both U2 snRNP and the SmR160/300 splicing coactivator with the dsx(GAA)₆ pre-mRNA (25). By contrast, in the presence of U1 snRNP, but absence of the ESE, these components bound weakly to this substrate. Taken together with the results of the present study, we propose that U1 snRNP promotes two distinct sets of interactions during ESE-dependent splicing. One set involves ESE-independent interactions that are required for the binding of U2AF-65 kDa to the polypyrimidine tract, which then promotes partial binding of U2 snRNP to the branch site. This set of interactions likely involves previously proposed cross-intron interactions mediated by the branch site-binding factor SF1/mBBP, which interacts with U2AF-65 kDa and is also required for the stable binding of U2 snRNP to the branch site (47, 48).

The other set of interactions promoted by U1 snRNP simultaneously requires the ESE and functions to further stabilize the binding of U2 snRNP to the branch site, and also promotes the association of SmR160/300 with the pre-mRNA (25). This set of interactions does not influence the binding of U2AF to the pre-mRNA. Although depletion of SmR160/300 or U2 snRNP weakens but does not prevent the association of the other component with the dsx(GAA)₆ pre-mRNA, these two components interact specifically (25). Thus, instead of promoting splicing complex formation through interactions mediated by the U2AF heterodimer, one or more ESE-associated components, including the SmR160/300 splicing coactivator, may promote splicing by interacting directly with the snRNP machinery of the spliceosome.

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