REPORT

3' splice site recognition in nematode *trans*-splicing involves enhancer-dependent recruitment of U2 snRNP

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ABSTRACT

Trans-splicing requires that 5' and 3' splice sites be independently recognized. Here, we have used mutational analyses and a sensitive nuclease protection assay to determine the mechanism of *trans*-3' splice site recognition *in vitro*. Efficient recognition of the 3' splice site is dependent upon both the sequence of the 3' splice site itself and enhancer elements located in the 3' exon. We show that the presence of three distinct classes of enhancers results in increased binding of U2 snRNP to the branchpoint region. Several lines of evidence strongly suggest that the increased binding of U2 snRNP is mediated by U2AF. These results expand the roles of enhancers in constitutive splicing and provide direct support for the recruitment model of enhancer function.

Keywords: exonic splicing enhancer; nematodes; SR proteins; *trans*-splicing; U2AF

INTRODUCTION

Trans-splicing is a required step in pre-mRNA processing in a variety of lower eukaryotes including the kinetoplastid protozoa and nematodes. In this reaction, the 5' exon (spliced leader) is supplied by a *trans*-splicing specific snRNP, the SL RNP, whereas the 3' exon is derived from a separately transcribed pre-mRNA (reviewed in Blumenthal, 1995; Nilsen, 1997). Of necessity, *trans*-splicing depends upon independent recognition of 5' and 3' splice sites and an efficient mechanism to juxtapose those sites.

Even though the splice site sequences themselves are similar in *trans*- and *cis*-splicing, evidence suggests that *trans*-splice site recognition is mechanistically distinct from the analogous process in *cis*-splicing. In this regard, *trans*-5' splice site engagement is determined by multiple features of the SL RNP. This RNP belongs to the Sm class of U snRNPs (Bruzik et al.,

1988; Thomas et al., 1988) and a functional Sm protein-binding site of specific sequence is an absolute requirement for function (Maroney et al., 1990; Denker et al., 1996). In addition to Sm proteins, the SL RNP contains two proteins (30 and 175 kDa) that are also required for SL RNP function (J.A. Denker, P.A. Maroney, C.M. Romfo, & T.W. Nilsen, in prep.). Several lines of evidence indicate that, upon assembly, the SL RNP engages U4/U6.U5 tri-snRNP independent of the presence of a 3' splice site (Maroney et al., 1996). It is clear that U1 snRNP, which plays a hierarchical role in *cis*-5' splice site recognition, does not perform this function in *trans*-splicing (Maroney et al., 1996; reviewed in Nilsen, 1997).

Although 5' splice site recognition in *trans*-splicing is beginning to be understood, information pertaining to 3' splice site recognition is relatively sparse. Systematic analysis of the *Caenorhabditis elegans* genome has revealed that *cis*- and *trans*-3' splice sites appear to be indistinguishable at the primary sequence level (Blumenthal & Steward, 1997). This similarity extends to functional analysis; Blumenthal and colleagues (Conrad et al., 1991) have shown that a *C. elegans cis*-3' splice site uncoupled from its associated 5' splice site can serve as a site of SL addition. We have made

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analogous observations in an *Ascaris* cell-free system (Maroney et al., 2000a). Indeed, experiments to date suggest that the requirements for a functional *trans*-3' splice site are remarkably simple; that is, absence of a 5' splice site in *cis*, an AG dinucleotide in an appropriate sequence context, and an AU-rich "outtron" (the RNA upstream of a *trans*-3' splice site) of sufficient length (Conrad et al., 1995).

Here, we have examined *trans*-3' site recognition in detail and find that the aforementioned "simple" requirements are necessary but not sufficient for efficient *trans*-splicing. In addition to sequences that comprise the 3' splice site itself, the extent of functional 3' splice site recognition (and *trans*-splicing) is determined by *cis*-acting elements within the 3' exon. These elements, which can be dispersed throughout the exon or present as discrete sequences, function as exonic splicing enhancers to promote stable U2 snRNP binding to the 3' splice site region. The data provide strong support for the recruitment model of splicing enhancer function and raise the interesting possibility that this mode of 3' splice site recognition may have arisen early in evolution (coincident with the emergence of *trans*-splicing), and was later coopted for use in regulated and/or alternative splicing in higher eukaryotes.

RESULTS AND DISCUSSION

In previous analyses of splicing in nematode extracts, we have characterized pre-mRNAs that are efficiently processed by either *cis*- or *trans*-splicing (Hannon et al., 1990, 1991). In light of the current understanding of 3' splice site recognition, we were surprised that mutational inactivation or removal of the 5' splice site from a particular *cis*-spliced pre-mRNA did not yield a molecule that could serve as an efficient SL acceptor. The availability of this construct afforded us the opportunity to identify features that were required for *trans*-splicing. Accordingly, we constructed a number of "mix and match" chimeric molecules comprised of portions of the *cis*-3' half molecule and portions of an efficiently *trans*-spliced molecule. When the *trans* outtron was fused with the *cis* exon, no improvement in splicing efficiency was observed (data not shown). However, when the *cis* outtron was affixed to the *trans* exon, the resulting molecule served as an efficient substrate for *trans*-splicing (Fig. 1A). Because the 3' splice site region itself is identical in both molecules, this result indicated that important determinants of *trans*-splicing efficiency may reside within an exon. In an attempt to localize the sequence elements that promoted *trans*-splicing, we performed scanning mutagenesis (10-base blocks) throughout the exon. However, no single mutation had a discernable phenotype, a result that suggested the presence of multiple dispersed (presumably redundant) elements throughout the exon (data not shown and see below). Indeed, such an arrangement of positively act-

ing exonic elements has been previously observed (Schaal & Maniatis, 1999).

Numerous studies in *cis*-splicing systems have shown that exonic sequence elements can function by promoting splice site recognition (reviewed in Blencowe, 2000; Graveley, 2000). Therefore, it seemed possible that the exon-specific effects we observed might be related to differential recognition of the 3' splice site. To test this possibility, we assessed the association of *trans*-acting factors with the 3' splice site region of splicing substrates containing the "*trans*" or "*cis*" exons. Engagement of the 3' splice site was measured by monitoring nuclease protection of molecules containing a single labeled phosphate between the AG of the 3' splice site (Maroney et al., 2000b).

When labeled "*trans*" substrate was mixed with pre-diluted extract and immediately digested with micrococcal nuclease, essentially no protection was observed (Fig. 1B, lane 1). However, when the same substrate was incubated with extract under splicing conditions prior to nuclease digestion, three prominent families of nuclease-resistant fragments were detected: fragment set I with a mean size of 48 nt, fragment set II with a mean size of 40 nt, and fragment set III with a mean size of 10 nt. Mapping via site specific RNase H digestion (Maroney et al., 2000b) revealed that all three fragment sets had a common 3' end ($\sim +2$ with respect to the 3' splice site). Thus, the difference in size of the fragment sets corresponded to the extent of intronic sequence protected; that is, -46 , -38 , and -8 , respectively.

Protection of the larger fragment sets (I and II) required functional U2 snRNP, because sequestration of the branchpoint interaction region of U2 with a complementary 2' Ome oligonucleotide prevented their appearance (Fig. 1B, lane 3; Fig. 1C, lane 4); oligonucleotides complementary to U1, U6, or SL RNAs had no effect (Fig. 1B,C). Accordingly, we conclude that protection of fragment sets I and II results from stable binding of U2 snRNP and associated factors to the substrate. The protected regions extend well upstream of the branchpoint (which is at -19 in this substrate; our unpubl. observation). Such extensive U2 snRNP-dependent protections have been previously observed (Ruskin & Green, 1985) and are consistent with the crosslinking studies of Reed and colleagues (Staknis & Reed, 1994; Gozani et al., 1996), which have documented a number of protein/RNA contacts both upstream and downstream of the branchpoint. At present, we do not know if fragment sets I and II are functionally distinct; however it seems likely that fragment set II is derived from fragment set I by nuclease nicking.

The small protected fragment set (III) is insensitive to inactivation of any snRNP. The pattern of nuclease-resistant species in fragment set III is remarkably similar to that observed upon binding of recombinant heterodimeric human U2AF to the same molecule (Wu

FIGURE 1. Exon-specific effects on *trans*-splicing and U2 snRNP binding. **A:** Uniformly labeled pre-mRNA substrates containing a naturally *trans*-spliced exon (closed box, lane 1) or a *cis*-3' exon (open box, lane 2) and the 3' portion of a *cis* intron (see text and Materials and Methods), were incubated for 30 min in *Ascaris* extract under splicing conditions as described (Yu et al., 1993). Following incubation, labeled RNAs were fractionated by denaturing PAGE and visualized by autoradiography. Intermediates and products of *trans*-splicing are indicated schematically. Lane M: marker DNA fragments of known size. **B:** The same pre-mRNAs as in **A** were uniquely labeled between the A and G of the 3' splice site exactly as described (Maroney et al., 2000b). Equal cpm of each substrate were incubated for 10 min under splicing conditions in the absence of any 2'Ome oligonucleotide (lanes 2 and 6) or in the presence of 2'Ome oligonucleotides complementary to U2 (lanes 3 and 7) or U6 (lanes 4 and 8). Following incubation, reactions were diluted and digested with micrococcal nuclease prior to fractionation on a 8% denaturing gel. For lanes 1 and 5, labeled substrate was added to prediluted extract and immediately digested with nuclease (see Materials and Methods). The sizes of DNA markers are indicated (lane M). **C:** The same specifically labeled pre-mRNA as in **B**, lanes 1–4, was incubated as in **B** in the absence (lane –) or presence of 2'Ome oligonucleotides complementary to the indicated U snRNAs. Experimental manipulations were as in **B**. **D:** Schematic representation of the boundaries of the nuclease protected fragment sets as indicated.

et al., 1999). This fact coupled with the results of site-specific photocrosslinking (data not shown) and mutational analysis (see below) supports the conclusion that protected fragment set III results from stable binding of heterodimeric U2AF to the 3' splice site.

In the accompanying article, Boukis and Bruzik (2001) have used a randomization/selection protocol (SELEX) to identify exonic sequence elements that can promote *trans*-splicing in vitro. Briefly, they introduced a randomized 18-nt sequence into a derivative of the "cis" 3' half molecule described above (see Fig. 1). Although the starting pool was an inefficient substrate for *trans*-splicing, several rounds of enrichment yielded discrete sequence elements that conferred a striking enhancement of *trans*-splicing (Boukis & Bruzik, 2001). We wished to determine if the elements identified by functional SELEX exerted their effects in a manner similar to the elements present in a naturally *trans*-spliced exon; that is, via promotion of 3' splice site recognition. Two classes of enhancers were examined; the first contains repeats of the sequence $^G/C$ GAC $^G/C$; the second contains sequence elements that resemble a 5' splice site

(Boukis & Bruzik, 2001). Figure 2 shows that the presence of either enhancer dramatically increased U2 snRNP binding relative to the enhancerless molecule; the level of U2 snRNP association is comparable to that seen with the naturally *trans*-spliced exon. In this regard, the pattern of resistant fragments due to U2 snRNP binding observed with this molecule is altered somewhat from that seen with our wild-type substrate (i.e., fragment sets I and II are replaced with a single fragment set I*). This difference is presumably due to the fact that the outtron sequences of the two molecules diverge upstream of position -42. It is unclear, however, how this sequence variation affects nuclease resistance.

Boukis and Bruzik (2001) have shown that the enhancement of *trans*-splicing conferred by the 5' splice site-like sequence is dependent upon U1 snRNP binding; that is, sequestration of the 5' end of U1 snRNA with an antisense oligonucleotide abrogated enhancement. Importantly, occlusion of the 5' end of U1 snRNA prevented U2 snRNP binding to the molecule containing the 5' splice site-like enhancer (Fig. 2, lane 18).

FIGURE 2. Distinct types of exonic enhancers promote U2AF and U2 snRNP binding. Four pre-mRNA substrates differing in exonic sequence were uniquely labeled between the A and G of the 3' splice site; the closed box indicates the "trans" exon as in Figure 1B,C, the open box represents the starting construct prior to functional SELEX (see text and Materials and Methods), the exon labeled SGACS contains the sequence (GGACCGACGCCGGACG) ~110 bases downstream from the 3' splice site and the exon labeled 5'SS contains a sequence resembling a 5' splice site (AGGUAAGU) at the same position (see text and Boukis & Bruzik, 2001). Each labeled substrate was incubated in *Ascaris* extract and subsequently digested with micrococcal nuclease as in Figure 1B,C. Incubations were without oligonucleotide pretreatment (lanes —) or following pretreatment with the indicated 2'Ome oligonucleotide. Fragment set indications are as in Figure 1. In lanes 6–20, fragment set I* replaces fragment sets I and II (see text). The sizes and positions of DNA markers are indicated.

These experiments further document the direct correlation between the ability of a 3' half-molecule to bind U2 snRNP and its ability to participate in *trans*-splicing. Furthermore, three distinct types of exonic elements can function to promote U2 snRNP binding.

There are striking similarities between the 3' exonic requirements for *trans*-splicing we observe and those previously established by Chiara and Reed (1995) in their studies of bimolecular splicing in mammalian extracts. They demonstrated that enhancers (either purine rich elements or a downstream 5' splice site) promoted formation of a stable complex at the 3' splice site and that such complexes could then participate in splicing. The parallels between 3' splice site engagement in natural and experimentally derived *trans*-splicing systems contrast with the quite distinct mechanisms of 5' splice site recognition in the two systems (see Denker et al., 1996 for discussion).

Having established that U2 snRNP binding was directly correlated with competence for *trans*-splicing, we sought to define further the mechanism of exon-dependent U2 snRNP association. We have previously demonstrated that binding of recombinant human U2AF to the 3' splice site of our substrate is dependent upon

the integrity of the AG dinucleotide (Wu et al., 1999). In concurrent studies, Zorio and Blumenthal (1999) showed that binding of *C. elegans* U2AF to a 3' splice site oligonucleotide was AG dependent. The nuclease protection data shown in Figures 1 and 2 were consistent with the possibility that U2AF binding preceded and was required for U2 snRNP association with the 3' splice site (see above). As shown in Figure 3, mutation of the AG dinucleotide to UC completely abolished splicing and, in parallel, abolished all nuclease protection. The most straightforward interpretation of these results is that the AG dinucleotide is required for U2AF binding, which in turn is required for U2 snRNP association.

The foregoing experiments established that several different exonic enhancer elements (either natural or obtained via functional selection) could promote stable recognition of the same 3' splice site. Considerable experimental evidence indicates that many enhancers can exert their effects by providing binding sites for one or more of the SR family of splicing factors (Blencowe, 2000; Graveley, 2000). To determine if stable U2 snRNP association was SR protein dependent, we took advantage of the fact that extracts prepared from early stage (two-cell) *Ascaris* embryos lack functional SR proteins

FIGURE 3. *Trans*-splicing as well as U2AF and U2 snRNP binding are AG dependent. In vitro splicing (**A**) and nuclease protection analysis (**B**) of pre-RNAs with a wild type (AG) or mutant (UC) 3' splice site. Splicing assays were as in Figure 1A except that the *trans*-splice acceptor was 3'-end labeled with [32 P]-pCp. Splicing products and intermediates are indicated schematically. **B:** Nuclease protection assays (as in Fig. 1B) were performed with the pre-mRNAs used in **A**. Wild-type substrate contained a unique label between the A and G of the 3' splice site; the mutant substrate contained a unique label between the U and C of the altered 3' splice site. Experimental manipulations were exactly as in Figure 1B.

(Sanford & Bruzik, 1999). Early stage extracts cannot catalyze *cis*- or *trans*-splicing, but both activities are restored upon addition of SR proteins prepared from later stage embryos (Sanford & Bruzik, 1999). When the nuclease protection assay was performed in two-cell extracts using the substrate containing the naturally *trans*-spliced exon, no resistant fragments were observed (Fig. 4, lanes 1–3). Strikingly however, U2AF as well as U2 snRNP-dependent protections were restored upon addition of purified active SR proteins (Fig. 4, lanes 4–6). Parallel analysis showed that splicing activity was fully reconstituted upon addition of the same preparation of SR proteins (data not shown). These results provide direct evidence that the exonic elements mediate their effects (directly or indirectly) through SR proteins. Furthermore, the fact that no stable binding of U2AF is observed in the absence of SR

proteins, but is readily apparent in their presence, provides a clear example of SR protein-dependent recruitment of U2AF.

Using a series of matched pre-mRNAs, we have shown that the efficiency of *trans*-splicing can be determined by exonic sequence content, and that efficiency of splicing is directly correlated with the ability of exonic elements to promote 3' splice site recognition. This recognition, as evidenced by the stable binding of U2AF and U2 snRNP, can be mediated by disperse (or large) elements, as in the naturally *trans*-spliced exon or by discrete sequence elements identified by functional SELEX. All of the elements appear to function via recruitment of U2AF, which in turn mediates U2 snRNP binding. In the case of the natural *trans*-spliced exon, SR proteins mediate U2AF/U2 snRNP recruitment.

Although we know that SR proteins are required for U2AF recruitment, it is important to note that we do not understand, in detail, the mechanism of this recruitment. It is possible that recruitment of heterodimeric U2AF is mediated by direct interaction between SR proteins and U2AF35 (e.g., Zuo & Maniatis, 1996). Alternatively, other proteins might act as bridges between U2AF and SR proteins. In this regard, using a novel nuclear retention assay, Blumenthal and colleagues have provided strong evidence that multiple factors are involved in 3' splice site identification in *C. elegans* (MacMorris et al., 1999; T. Blumenthal, pers. comm.).

We also do not (and cannot) know whether enhancer-dependent 3' splice site recognition is general to all cases of *trans*-splicing; such a determination would require an extensive survey. It may be possible, for example, that certain 3' splice sites have a high intrinsic affinity for U2AF. Indeed, Zorio and Blumenthal (1999) have measured, via crosslinking, a direct interaction between U2AF and an oligonucleotide comprised of the 3' splice site consensus sequence. However, it is difficult to correlate the efficiency of crosslinking with splicing. Furthermore, as noted above, from genomic analyses, there is no indication of signature sequences that would differentiate between "strong" and "weak" *trans*-splice acceptor sites. Although our analysis is limited to a few substrates, there is evidence that enhancer mediated 3' splice site recognition may be generalizable. Specifically, Ullu and colleagues have shown that exonic elements can play a major role in determining *trans*-splicing efficiency in trypanosomes (López-Estraño et al., 1998). These observations, coupled with the fact that trypanosomatids possess SR proteins (Ismaili et al., 1999), make it tempting to speculate that 3' splice site recognition in these organisms is mediated by SR-dependent exonic splicing enhancers. If true, this pathway would have emerged quite early in evolution, as kinetoplastid protozoans are among the most deeply branched eukaryotes (Sogin et al., 1989).

FIGURE 4. Recruitment of U2AF and U2 snRNP requires active SR proteins. Nuclease protection analysis of site specifically labeled wild-type pre-mRNA (Fig. 3A, wt) was performed in 2-day *Ascaris* extract that lacks active SR proteins (see text) either in the absence (mock, lanes 1–3) or presence of 0.5 μ g of purified active SR proteins (lanes 4–6). Experimental manipulations were identical to those in Figure 1B,C.

MATERIALS AND METHODS

Pre-mRNA substrates

All substrates were derived from either a naturally trans-spliced pre-mRNA or a cis-spliced intron (Hannon et al., 1990, 1991) and its accompanying 3' exon. The substrate used in Figure 1A, lane 1, contained the 3'-most 79 nt of the cis intron fused to the trans exon. The substrate used in Figure 1A, lane 2, contained the same 79-nt intronic sequence fused to the cis 3' exon. Substrates used in Figure 2 included a shortened derivative of the same cis intron/exon combination (lanes 6–10) and two pre-mRNAs derived by functional SELEX (Boukris & Bruzik, 2001; see legend to Fig. 2). For analysis of splicing, RNAs were labeled with either [α - 32 P]-GTP (Fig. 1) or [32 P]-pCp (Fig. 3A). Site-specific labeling at the 3' splice site followed the procedure described by Maroney et al. (2000b). Briefly, trace-labeled pre-mRNAs were specifically cleaved with chimeric 2'Ome/DNA oligonucleotides. Resultant 3' half molecules were dephosphorylated with CIAP and subsequently rephosphorylated with polynucleotide kinase and [γ - 32 P]-ATP. The labeled 3' half molecules were then rejoined to the 5' half molecules using splinted ligation (Moore & Sharp, 1992).

In vitro splicing and nuclease protection analysis

Splicing assays in *Ascaris* extracts were carried out as previously described (Yu et al., 1993). Nuclease protection assays were performed exactly as described in detail (Maroney et al., 2000b). For snRNP inactivation, 2'Ome oligonucleotides corresponding to bases 1–14 of U1 snRNA, bases 29–44 of U2 snRNA, bases 77–95 of U6 snRNA, or bases 22–42 of the SL RNA were added to reactions as indicated in the figure legends. All inactivations were performed by preincubating the extract for 5 min at room temperature prior to the addition of labeled RNA.

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