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Purine-rich enhancers function in the AT-AC pre-mRNA splicing pathway and do so independently of intact U1 snRNP

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ABSTRACT

A rare class of introns in higher eukaryotes is processed by the recently discovered AT-AC spliceosome. AT-AC introns are processed inefficiently in vitro, but the reaction is stimulated by exon-definition interactions involving binding of U1 snRNP to the 5′ splice site of the downstream conventional intron. We report that purine-rich exonic splicing enhancers also strongly stimulate sodium channel AT-AC splicing. Intact U2, U4, or U6 snRNAs are not required for enhancer function or for exon definition. Enhancer function is independent of U1 snRNP, showing that splicing stimulation by a downstream 5′ splice site and by an exonic enhancer differ mechanistically.

Keywords: AT-AC intron; exon definition; exonic splicing enhancer (ESE); pre-mRNA splicing; U1 snRNP

INTRODUCTION

AT-AC splicing is catalyzed by a minor spliceosome with at least four snRNA constituents distinct from those present in the major spliceosome (reviewed in Nilsen, 1998). AT-AC introns were first recognized on the basis of their distinctive splice-site consensus sequences (Jackson, 1991). Initially, only four examples of phylogenetically conserved AT-AC introns were found in vertebrate or invertebrate genes (Hall & Padgett, 1994; Tarn & Steitz, 1996a). More recently, extensive sequence compilations showed that AT-AC introns are more abundant and are present in all higher eukaryotes (Sharp & Burge, 1997; Tarn & Steitz, 1997; Wu & Krainer, 1997). The U12 snRNA was recently shown to be essential for AT-AC splicing both in vivo (Hall & Padgett, 1996) and in vitro (Tarn & Steitz, 1996a; Wu & Krainer, 1996). The U11 snRNA is present in spliceosomes assembled in vitro (Tarn & Steitz, 1996a) and can be crosslinked to an AT-AC intron 5′ splice site (Yu & Steitz, 1997). U11 was also shown to base pair with an AT-AC intron 5′ splice site in vivo (Kolossova & Padgett, 1997). These findings confirmed the predicted roles of U11 and U12 snRNAs (Hall & Padgett, 1994). The U4atac and U6atac snRNAs, two novel snRNAs isolated from an in vitro-assembled AT-AC spliceosome, were shown to be essential for processing of the AT-AC intron of proliferating cell nucleolar antigen (P120) in vitro (Tarn & Steitz, 1996b). These two snRNAs are also essential for in vitro splicing of an AT-AC intron in the voltage-gated skeletal muscle sodium channel α subunit (SCN4A) pre-mRNA (Wu & Krainer, 1997). Recently, U6atac was shown to recognize the AT-AC 5′ splice site by base pairing in vivo (Incorvaia & Padgett, 1998).

In the major splicing pathway, the pre-mRNA *cis*-acting signals that help define the correct exon–intron boundaries include the 5′ splice site, 3′ splice site, and branch site elements, as well as exonic or intronic splicing enhancer or silencer elements (reviewed in Black, 1995). Individual signals are degenerate in sequence, but collectively they allow precise removal of introns by the spliceosome. Accurate splicing appears to require a fine balance between intrinsic splice-site strengths and modulation by the auxiliary elements that surround the splice sites, especially in pre-mRNAs that undergo alternative splicing (reviewed in Cáceres & Krainer, 1997). Exonic splicing enhancers (ESEs) are frequently found in the exons downstream of the intron whose splicing they influence (reviewed in Hertel et al., 1997).

The most studied class of ESE is composed of purine-rich sequences (Lavigueur et al., 1993; Sun et al., 1993; Watakabe et al., 1993; Ramchatesingh et al., 1995). The purine-rich ESEs are recognized by members of

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the SR protein family, a group of essential pre-mRNA splicing factors with characteristic arginine/serine C-terminal repeats (RS domain) and one or two RNA-recognition motifs (Lavigueur et al., 1993; Sun et al., 1993; Staknis & Reed, 1994; Ramchatesingh et al., 1995; Achsel & Shimura, 1996; Yeakley et al., 1996). SR proteins help recognize weak splice sites and promote spliceosome assembly (Staknis & Reed, 1994) in part through interactions with 3′ splice-site components (Wang et al., 1995; Valcárcel et al., 1996; Zuo & Maniatis, 1996). Enhancer recognition and complex assembly are sufficiently strong to promote *trans*-splicing via 5′ and 3′ splice sites located on separate RNA molecules (Bruzik & Maniatis, 1995; Chiara & Reed, 1995).

Little is known about splice-site recognition and commitment in the AT-AC pathway, other than the high conservation of sequence near the intron ends and the base pairing with the low abundance U11, U12, and U6atac snRNAs (reviewed in Nilsen, 1998). In the major pathway, short exons often appear to be defined as units prior to the pairing of splice sites, in a process known as exon definition (reviewed in Berget, 1995). We showed previously that exon definition is also relevant to the AT-AC splicing pathway; thus, a downstream conventional 5′ splice site increases the efficiency of AT-AC splicing, and this effect is dependent on the integrity of the U1 snRNP (Wu & Krainer, 1996). In the present study, we show that purine-rich enhancers can promote AT-AC splicing as well, indicating that the mechanisms of enhancer function and exon definition are compatible with the two different kinds of components that are responsible for 3′ splice site recognition in the conventional and the AT-AC splicing pathways. The fact that U1, U2, U4, and U6 snRNAs are not required for the basal AT-AC splicing reaction (Tarn & Steitz, 1996a,b; Wu & Krainer, 1996, 1997) provides an opportunity for investigating whether these snRNAs play a role in enhancer function, as previously suggested for the U1 snRNA (Watakabe et al., 1993; Staknis & Reed, 1994; Stark et al., 1998). We have tested the effects of cleavage, blockage, or depletion of the major snRNAs on enhancer function and exon definition in the context of AT-AC splicing. The results reveal that splicing stimulation by a downstream 5′ splice site or by a downstream ESE have a different mechanistic basis.

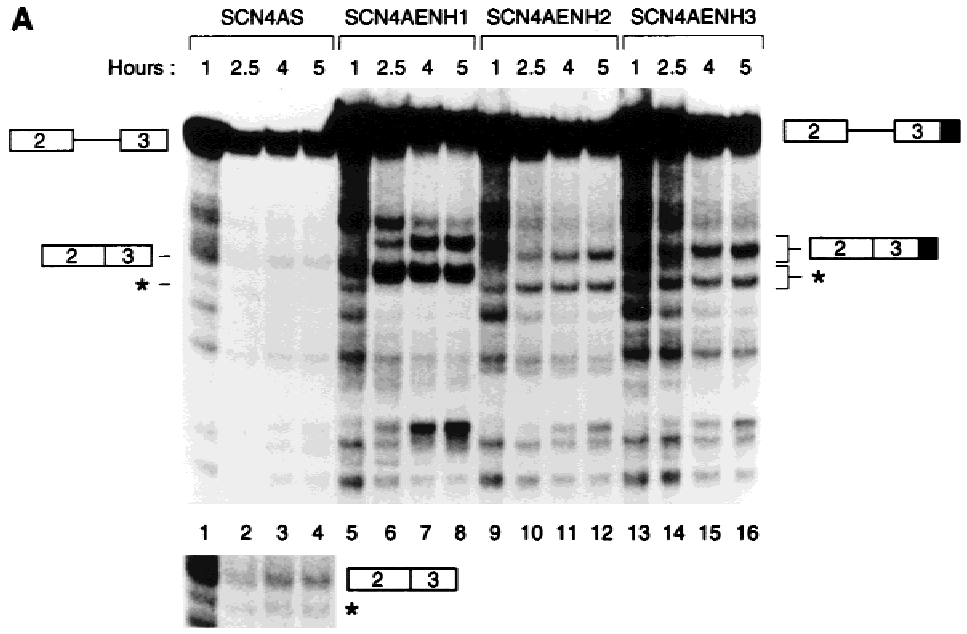
RESULTS AND DISCUSSION

Purine-rich enhancers stimulate AT-AC pre-mRNA splicing in vitro

Exon definition and splicing enhancers are thought to influence splicing efficiency by a very similar mechanism (Watakabe et al., 1993; Staknis & Reed, 1994; Chiara & Reed, 1995; Wang et al., 1995; Achsel & Shimura, 1996). Both effects contribute to the selection of upstream splice sites and appear to involve SR proteins (reviewed in Berget, 1995; Black, 1995; Valcárcel et al., 1995; Manley & Tacke, 1996; Cáceres & Krainer, 1997). A downstream conventional 5′ splice site promotes splicing of the AT-AC intron 2 of the voltage-gated skeletal muscle sodium channel α subunit (SCN4A) pre-mRNA (Wu & Krainer, 1996). Purine-rich enhancers have been shown to function with a wide variety of substrates in the major splicing pathway. To determine whether they can also function in the context of the AT-AC splicing pathway, we prepared sodium channel pre-mRNA derivatives containing heterologous purine-rich enhancer elements, and compared their splicing efficiencies to that of the parent pre-mRNA (Fig. 1A). The chosen motifs are present in the natural enhancers previously identified in a wide variety of cellular and viral genes (Fig. 1B).

The purine-rich sequences placed at the end of the 90-nt exon 3 greatly enhanced splicing of the AT-AC intron 2 in HeLa cell nuclear extract (Fig. 1A). The basal AT-AC splicing reaction with the SCN4AS substrate (Wu & Krainer, 1997) is very weak (Fig. 1A, lanes 1–4), although the spliced products are visible in the longer exposure (Fig. 1A, bottom). As is commonly observed with pre-mRNAs that splice inefficiently, there was considerable RNA degradation during the incubation. The splicing stimulation by inclusion of the enhancer elements is not simply due to extending the length of the downstream exon, because splicing of the SCN4AM pre-mRNA, which also has an extension (GGAUCC GAAUU), is not as efficient as in the presence of the enhancers (data not shown and see below). The enhancers stimulated AT-AC splicing to different extents (Fig. 1A, lanes 5–8, 9–12, and 13–16). The SCN4AENH1 pre-mRNA spliced most efficiently, suggesting an additive effect between the two elements in the bipartite enhancer. Quantitation of the data showed that the splicing efficiency of SCN4AENH1, SCN4AENH2, and SCN4AENH3 pre-mRNAs increased seven-, three-, and fourfold, respectively, compared to the enhancerless SCN4AS pre-mRNA. SCN4AENH1, SCN4AENH2, and SCN4AENH3 pre-mRNAs spliced more rapidly than SCN4AS pre-mRNA. The AT-AC spliced product is barely detectable in the case of the SCN4AS pre-mRNA after 2.5 h of incubation, even in the longer exposure. In contrast, splicing can be readily detected at this time point with the enhancer-containing pre-mRNAs. The enhancers also stimulated the previously described aberrant splicing reaction involving a pair of cryptic conventional splice sites (Wu & Krainer, 1996). The stimulation of this pathway was even stronger than that of the AT-AC pathway, perhaps because the cryptic 3′ splice site is closer to the enhancer elements.

The catalytic core of the basal AT-AC spliceosome is thought to be very similar to that of the major spliceosome (Tarn & Steitz, 1996b; reviewed in Nilsen, 1996).



B

SCN4AENH1: **GGAAGGAUCCGGAAGAAUU**

SCN4AENH2: **GGAAGGAUC**

SCN4AENH3: **GGAGAUGGGAGGAUC**

FIGURE 1. Heterologous purine-rich exonic elements enhance AT-AC pre-mRNA splicing in vitro. **A:** In vitro splicing time course in Hela cell nuclear extract. The SCN4AS pre-mRNA (lanes 1–4) is truncated at the end of exon 3. The SCN4AENH1 (lanes 5–8), SCN4AENH2 (lanes 9–12), and SCN4AENH3 (lanes 13–16) pre-mRNAs have additional sequences following exon 3, which are shown in **B**. Capped, in vitro-transcribed, ^{32}P-labeled pre-mRNAs were incubated under optimized splicing conditions for the indicated times and analyzed by urea-PAGE and autoradiography. The structure and electrophoretic mobilities of the pre-mRNAs and spliced mRNAs are indicated schematically on each side. The solid black square at the end of exon 3 indicates the placement of the heterologous purine-rich enhancers. Aberrantly spliced mRNAs, arising from use of conventional cryptic 5′ and 3′ splice sites (Wu & Krainer, 1996), are indicated by an asterisk. A longer exposure that shows the spliced products obtained with the SCN4AS pre-mRNA is shown at the bottom. **B:** Nucleotide sequence of the enhancer elements. These sequences were joined to the 3′ end of exon 3 of the voltage-gated skeletal muscle sodium channel α subunit gene (SCN4A); the first 3 nt, GGA, are part of the natural 3′ end of exon 3, and the distance to the upstream 3′ splice site is 87 nt. Purines are shown in bold. Two pyrimidines at the end of the transcripts derived from the restriction site are also shown. The motif introduced into the SCN4AENH2 pre-mRNA represents enhancer sequences present in the following exons: the last membrane isoform-specific exon (M2) of mouse immunoglobin μ (IgM) (Watakabe et al., 1993); exon 5 of bovine growth hormone (bGH) (Sun et al., 1993); the last exon of avian sarcoma-leukosis virus (ASLV) env protein (Katz & Skalka, 1990; Fu et al., 1991); exon 3 of human hypoxanthine-guanine phosphoribosyltransferase (hprt) (Steingrimsdottir et al., 1992); and exon 4 of *Drosophila doublesex* (Lynch & Maniatis, 1995). The SCN4AENH1 pre-mRNA has a bipartite enhancer. The 5′ portion of this enhancer is the same as the enhancer of the SCN4AENH2 pre-mRNA. The 3′ portion includes sequences present in the natural enhancers of the following exons: exon 5 of chicken cardiac troponin T (cTNT) (Ramchatesingh et al., 1995); exon 3 of the rat calcitonin/CGRP gene (Yeakley et al., 1993); exon EDIIIA of human fibronectin (hFN) (Mardon et al., 1987; Lavigueur et al., 1993); the second coding exon of human immunodeficiency virus (HIV) tat-rev (Amendt et al., 1995; Staffa & Cochrane, 1995); the ASLV env last exon; and exon 3 of equine infectious anemia virus (EIAV) tat/rev (Gontarek & Derse, 1996). The SCN4AENH3 pre-mRNA includes enhancer sequences derived from the following exons: exon 3 of human hprt; exon EDIIIA of human FN; exon 5 of cTNT; exon 5 of human caldesmon (Humphrey et al., 1995); exon 8 of rat β-tropomyosin (TM) (Helfman et al., 1988); second coding exon of HIV tat-rev.

AT-AC pre-mRNA splicing requires the minor U11, U12, U4atac, and U6atac snRNAs, which appear to play analogous roles to those of the major U1, U2, U4, and U6 snRNAs in the major splicing pathway (reviewed in Nilsen, 1998). Exon definition and splicing enhancers markedly affect both AT-AC and conventional splicing. The SR proteins have been strongly implicated in splicing enhancer recognition and function. The three heterologous enhancers used in this study include sequences present in the enhancers from a wide variety of cellular genes, including IgM, hFN, bGH, *doublesex*, cTNT, caldesmon, calcitonin/CGRP, β-TM, and hprt, as well as of viral genes from ASLV, HIV, and EIAV, and are also present in in-vitro-selected sequences that are recognized by SR proteins (Tacke & Manley, 1995; Liu et al., 1998) (abbreviations are explained in the legend

to Fig. 1). The natural enhancers from hFN (Lavigueur et al., 1993), bGH (Sun et al., 1993), ASLV (Staknis & Reed, 1994), *doublesex* (Lynch & Maniatis, 1995), cTNT (Ramchatesingh et al., 1995), EIAV (Gontarek & Derse, 1996), and calcitonin/CGRP (Yeakley et al., 1996) have been shown to interact specifically with SR proteins. Exonic splicing enhancers are thought to facilitate 3′ splice-site recognition by specific binding to SR proteins, which in turn promote the binding of components at the upstream 3′ splice site through protein–protein interactions (Wang et al., 1995; Zuo & Maniatis, 1996). The finding that purine-rich enhancers function in both minor and major splicing pathways suggests that at least some of the functions of SR proteins are relevant to the AT-AC splicing pathway.

Putative enhancer elements in natural AT-AC pre-mRNA exons

Although we showed that purine-rich splicing enhancers can function in the context of an AT-AC intron, authentic enhancers that naturally function in this context remain to be identified. We scanned the sequences of the exons located immediately downstream of known AT-AC introns (Wu & Krainer, 1997) and found one or more purine-rich elements in several of these exons, including exon 7 of the human P120 gene, exon 8 of the human CMP gene, exon 7 of the human GT335 gene, exon 10 of the mouse CDK5 gene, exon 7 of the mouse Rep-3 gene, exon 16 of the mouse HPS gene, and exon 7 of a *Xenopus* TFIIS gene (data not shown). These purine runs are all located near the corresponding upstream AT-AC 3′ splice site (6–136 nt), well within the previously established limit for enhancer function in conventional splicing (~300 nt)(Lavigueur et al., 1993; Lynch & Maniatis, 1995). Several of these elements match the sequences of known purine-rich enhancers (Fig. 1B), and therefore, at least some of them might function as natural AT-AC splicing enhancers, although this remains to be tested. The SCN4A exon 3, present in the substrates used in this study, lacks such a sequence, which is consistent with its strong response to inclusion of a heterologous enhancer. As with conventional splicing enhancers, natural AT-AC splicing enhancers may be present at other exonic or intronic locations, and they may not always be purine rich (Tian & Maniatis, 1994; Tian & Kole, 1995; Coulter et al., 1997; Liu et al., 1998).

Intact U1, U2, U4, and U6 snRNAs are not required for purine-rich enhancer function

Whether the major spliceosomal snRNAs are involved in splicing enhancement, that is, in recognition of purine-rich enhancers or in bridging between enhancer-binding factors and conserved intron elements, is difficult to address rigorously in the context of the major splicing pathway because of the requirement for these snRNAs in the basal splicing reaction. U1 snRNP has been shown to play a role in exon definition in both the major and minor splicing pathways, and it does so by binding to the downstream conventional 5′ splice site (Robberson et al., 1990; Kuo et al., 1991; Wu & Krainer, 1996). A role for an snRNA in enhancer recognition has previously been suggested in the case of U1 (Watakabe et al., 1993; Staknis & Reed, 1994; Stark et al., 1998). Because the basal AT-AC splicing reaction does not require the major snRNAs, except for the U5 snRNA, nor is inactivation of the major pathway required in our in vitro system, we have exploited these unique snRNA requirements to investigate whether the snRNAs that function exclusively in the conventional pathway are required for enhancer function in the context of AT-AC splicing.

We used oligonucleotide-directed RNase H cleavage to inactivate the major snRNAs (Fig. 2). U2-, U6-, or U4-specific oligonucleotides did not inhibit SCN4AENH1 AT-AC splicing (Fig. 2, lanes 1, 5–7, 11–13, and 17–19). The same treatments likewise had no effect on exon definition (Wu & Krainer, 1997). Cleavage of the 5′ end of U1 snRNA did not inhibit SCN4AENH1 AT-AC splicing (Fig. 2, lanes 1–4), in contrast to the inhibition of exon definition by the same treatment (Wu & Krainer, 1996). However, U1-, U2-, U4-, or U6-specific oligonucleotides strongly inhibited splicing via the conventional cryptic splice sites, providing useful internal controls. U12-, U6atac-, or U4atac-specific control oligonucleotides completely inhibited SCN4AENH1 AT-AC splicing, but had no inhibitory effect on the cryptic splicing pathway, as expected (Fig. 2, lanes 8–10, 14–16, and 20–21). Very similar effects were obtained with the SCN4AENH2 and SCN4AENH3 pre-mRNAs (data not shown). In addition, different oligonucleotides complementary to other regions of U2, U4, and U6 snRNAs gave the same inhibition profiles with these substrates. We conclude that intact U1, U2, U4, and U6 snRNAs are not required for splicing activation by purine-rich enhancers.

Although U6 may be involved in exon definition (Hwang & Cohen, 1996), cleavage of U6 snRNA does not inhibit splicing of an AT-AC pre-mRNA with a conventional downstream 5′ splice site (Wu & Krainer, 1997). A role for U6 in this process may be more apparent if the downstream 5′ splice site has poor complementarity to U1 snRNA (Tarn & Steitz, 1994; Crispino & Sharp, 1995; Hwang & Cohen, 1996). Our previous results suggested that U4 and U2 snRNAs are not required for exon definition.

Mechanistic difference between enhancer function and exon definition

Cleavage of U2, U4, and U6 snRNAs does not inhibit AT-AC splicing with either the SCN4AENH1 pre-mRNA,

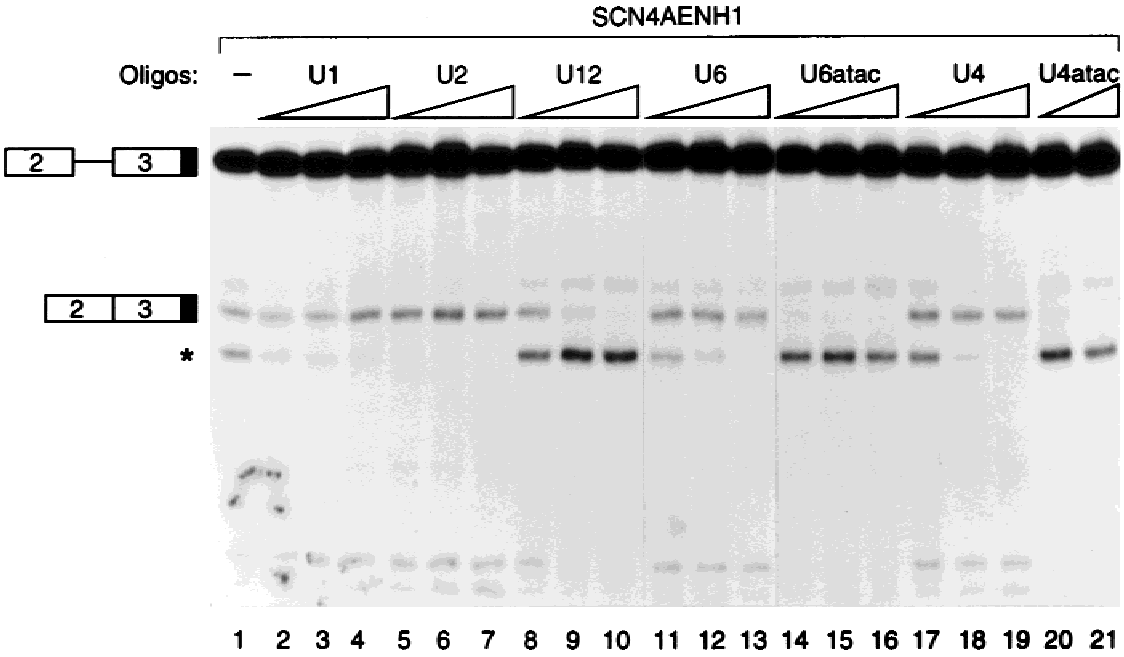


FIGURE 2. Purine-rich enhancer function does not require intact U1, U2, U4, or U6 major snRNAs. The major or minor spliceosomal snRNPs were inactivated by cleavage of their snRNA moieties with RNase H in the presence of complementary oligonucleotides. The nuclear extract was preincubated under splicing conditions for 15 min in the absence (lane 1) or presence of oligonucleotides specific for U1 (lanes 2–4), U2 (lanes 5–7), U12 (lanes 8–10), U6 (lanes 11–13), U6atac (lanes 14–16), U4 (lanes 17–19) or U4atac (lanes 20–21). The final oligonucleotide concentration was 0.33 μM (lanes 2, 5, 8, 11, 14, 17, and 20), 1.31 μM (lanes 3, 6, 9, 12, 15, 18, and 21), or 5.25 μM (lanes 4, 7, 10, 13, 16, and 19). SCN4AENH1 pre-mRNA was added to each reaction and incubation was continued for 6 h. Note that the exposure shown is shorter than that in Figure 1, and that the preincubation step results in a slight decrease in splicing efficiency.

which has a heterologous enhancer, or with the SCN4A pre-mRNA, which includes the downstream conventional 5′ splice site (Fig. 2; Wu & Krainer, 1997). In contrast, cleavage of the 5′ terminus of U1 snRNA inhibits SCN4A AT-AC splicing (Wu & Krainer, 1996), but has no effect on AT-AC splicing with the SCN4AENH1 pre-mRNA (Fig. 2). This observation reveals a clear difference between enhancer function and exon definition. As shown above, enhancer function does not require the 5′ terminus of U1 snRNA. However, this does not exclude a role for the rest of the U1 snRNP particle.

Previous studies showed that U1 snRNA lacking the 5′ terminus can still function in splicing of chimeric pre-mRNAs in which the 5′ splice site region of an adenovirus major late pre-mRNA is replaced by spliced leader sequences from *Leptomonas collosoma* or *Caenorhabditis elegans* (Bruzik & Steitz, 1990; Seiwert & Steitz, 1993). Antisense 2′-*O*-methyl oligonucleotides complementary to the 5′ terminus of U1 snRNA, which do not confer sensitivity to RNase H, block U1 snRNP function by a different mechanism—sequestration and/or steric hindrance—and can thus uncover additional U1 functions (Barabino et al., 1990; Seiwert & Steitz, 1993). We used this approach to debilitate U1 snRNP in HeLa nuclear extract (Fig. 3). β-Globin splicing was strongly inhibited (Fig. 3, lanes 1–5). AT-AC splicing of the SCN4A pre-mRNA was partially inhibited, reflecting a loss of exon definition, while splicing via the cryptic splice sites was completely inhibited (Fig. 3, lanes 6–10). These results are consistent with the inhibition of SCN4A AT-AC splicing by RNase H cleavage of the U1 snRNA 5′ end (Wu & Krainer, 1996). With the SCN4AM pre-mRNA, basal AT-AC splicing was not inhibited, and was in fact slightly stimulated, except at the highest oligonucleotide concentration, while cryptic splicing was inhibited (Fig. 3, lanes 11–15). The stimulation probably results from inhibition of the competing splicing reaction via the cryptic splice sites (Tarn & Steitz, 1996a; Wu & Krainer, 1997). Finally, AT-AC splicing of the enhancer-containing SCN4AENH1 pre-mRNA was stimulated by U1 debilitation, while cryptic splicing was completely inhibited (Fig. 3, lanes 16–20). The same 2′-*O*-methyl oligonucleotide had analogous effects with the SCN4AS pre-mRNA (which lacks the downstream 5′ splice site) and with the enhancer-containing SCN4AENH2 and SCN4AENH3 pre-mRNAs (data not shown). We conclude that exon definition is abrogated when U1 snRNP is debilitated, whereas enhancer function is not inhibited.

To address the possibility that U1 snRNP particles with a blocked U1 snRNA 5′ terminus retain an activity required for enhancer function, we depleted U1 snRNP from nuclear extract using antisense affinity chromatography with a biotinylated 2′-*O*-methyl oligonucleo-

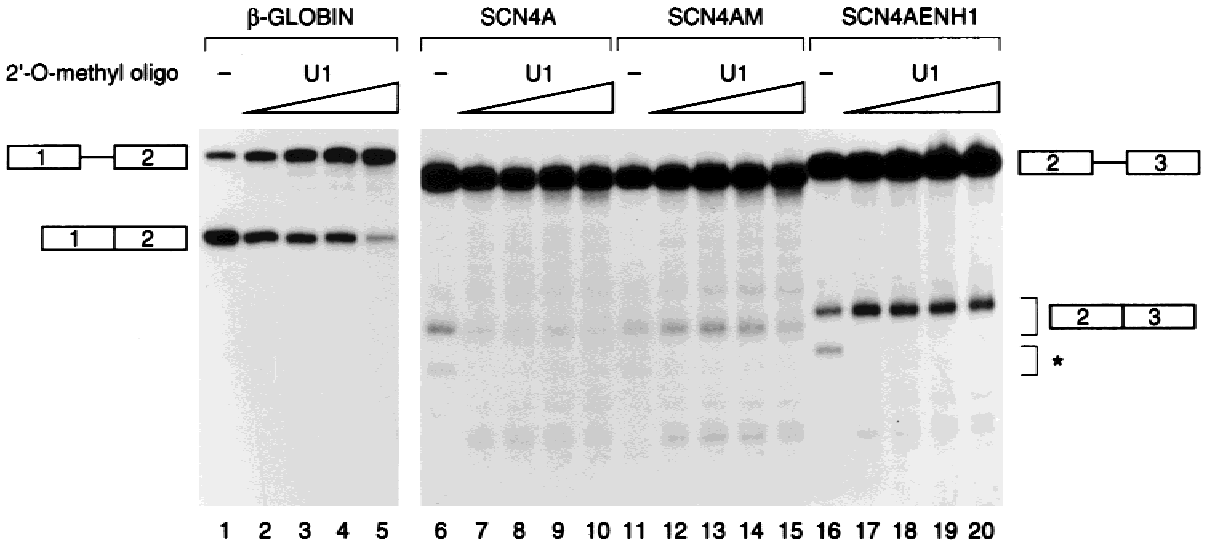


FIGURE 3. Effect of U1 snRNP debilitation on conventional and AT-AC splicing, exon definition, and enhancer function. Nuclear extract was preincubated in the absence (lanes 1, 6, 11, and 16) or presence (lanes 2–5, 7–10, 12–15, and 17–20) of a U1-specific 2′-*O*-methyl oligonucleotide at a final concentration of 2.63 μM (lanes 2, 7, 12, and 17), 5.25 μM (lanes 3, 8, 13, and 18), 10.5 μM (lanes 4, 9, 14, and 19), or 21.0 μM (lanes 5, 10, 15, and 20) under splicing conditions for 10 min. β-Globin (lanes 1–5), SCN4A (which has an AT-AC intron and includes the downstream conventional 5′ splice site; lanes 6–10), SCN4AM (which has a mutant downstream 5′ splice site; lanes 11–15), or SCN4AENH1 pre-mRNA (which has a heterologous enhancer; lanes 16–20), was added and the reactions were further incubated for 6 h.

tide (Barabino et al., 1990). In U1-depleted extracts, the control β-globin splicing reaction was strongly inhibited, compared to mock-depleted extracts (Fig. 4A, lanes 1 and 2). The same treatment partially inhibited SCN4A AT-AC splicing, and also inhibited splicing via the cryptic splice sites (Fig. 4A, lanes 3 and 4). With the SCN4AM pre-mRNA, AT-AC splicing was not inhibited, and was in fact slightly stimulated—as it was in the oligonucleotide inhibition experiments (Fig. 3)—but splicing via the cryptic conventional splice sites was inhibited (Fig. 4A, lanes 5 and 6). We note that SCN4A pre-mRNA splicing in U1-depleted extracts was reproducibly weaker than SCN4AM pre-mRNA splicing under the same conditions (Fig. 4A, lanes 3 and 5). This difference suggests that residual U1 snRNP directs exon definition with the SCN4A pre-mRNA, but does so poorly, whereas the SCN4AM pre-mRNA, which lacks the U1 binding site, is spliced via intron definition. Finally, SCN4AENH1 cryptic splicing was completely inhibited in the U1-depleted extracts, but AT-AC splicing was unaffected, compared to the mock-treated extract (Fig. 4A, lanes 7 and 8). These results once again reveal a difference in the requirement for U1 snRNP between enhancer function and exon definition.

Six sets of independent AT-AC splicing reactions were carried out using U1-depleted and mock-depleted extracts, and the resulting splicing efficiencies were measured (Fig. 4B). The strong inhibition of β-globin in U1-depleted versus mock-depleted extracts was reproducibly observed, and a statistically significant twofold reduction in SCN4A pre-mRNA AT-AC splicing was also seen. U1 depletion had no significant effect on AT-AC splicing in the absence of a wild-type downstream 5′ splice site (SCN4AM pre-mRNA) or in the presence of an enhancer (SCN4AENH1 pre-mRNA). Northern blotting with a U1-specific probe and a control U2-specific probe showed specific and extensive depletion of U1 snRNA (Fig. 4C, lanes 1 and 2). We do not know if the residual levels of β-globin splicing result from the trace levels of remaining U1 snRNP, or represent inefficient splicing via a previously proposed U1-independent pathway (Crispino et al., 1994; Tarn & Steitz, 1994).

Purine-rich enhancers stimulate AT-AC splicing independently of U1 snRNP

U1 snRNA was the first component shown to bind to vertebrate exonic splicing enhancers (Watakabe et al., 1993; Staknis & Reed, 1994; Stark et al., 1998; reviewed in Valcárcel et al., 1995; Manley & Tacke, 1996). The 5′ terminus of U1 snRNA was crosslinked to the IgM exon 2 purine-rich enhancer, suggesting that U1 snRNP may recognize enhancers by base pairing (Watakabe et al., 1993). U1 is also present in a complex assembled on an ASLV RNA containing an enhancer (Staknis & Reed, 1994). Finally, RNA affinity chromatography of an S100 extract with an enhancer-containing cTNT exon resulted in binding of U1 snRNA; this binding was stimulated by SR proteins and was largely dependent on the 5′ terminus of U1 (Stark et al., 1998). These studies suggested that U1 plays a role in enhancer recognition and/or function.

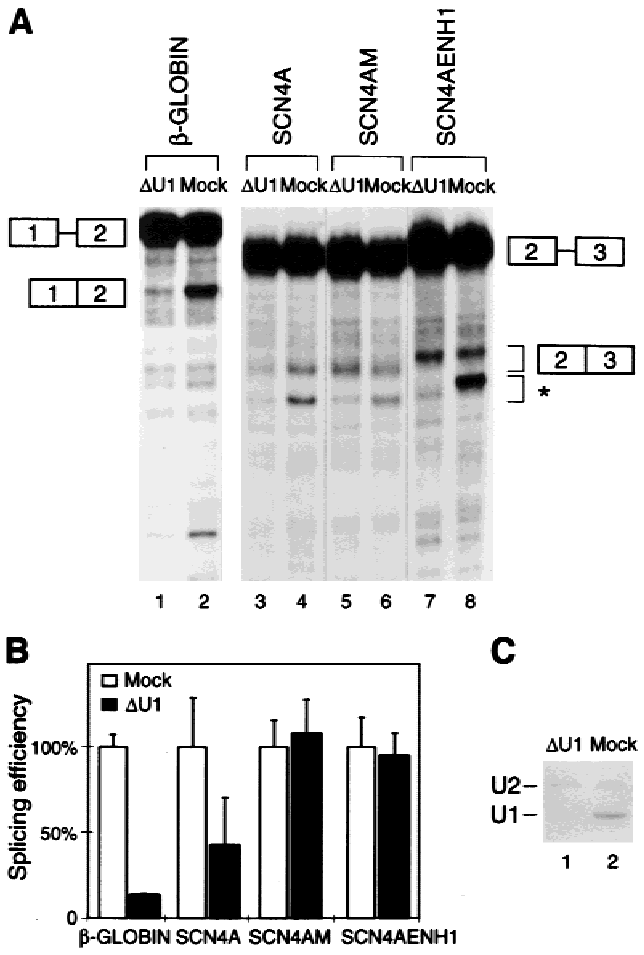


FIGURE 4. Effect of U1 snRNP depletion. **A:** Splicing of β-globin (lanes 1 and 2), SCN4A (lanes 3 and 4), SCN4AM (lanes 5 and 6), and SCN4AENH1 (lanes 7 and 8) pre-mRNAs in U1-depleted nuclear extract (lanes 1, 3, 5, and 7) or mock-depleted nuclear extract (lanes 2, 4, 6, and 8). **B:** Quantitation of splicing efficiencies in U1-depleted extracts. Six sets of splicing reactions were carried out for each AT-AC pre-mRNA, as in **A**, with U1-depleted extracts (black bars) and mock-depleted extracts (white bars). The β-globin pre-mRNA control was done twice. Splicing efficiency was normalized to the level obtained for each pre-mRNA in mock-depleted extract, which was set at 100%. **C:** Extent of depletion of U1 snRNP. Northern blot of U1-depleted extract (lane 1) and mock-depleted extract (lane 2) using U1- and U2-specific probes.

The proposed mechanisms of exon definition and enhancer function are closely related (Staknis & Reed, 1994; Berget, 1995; Wang et al., 1995). Both processes facilitate spliceosome assembly around short exons and involve bridging via SR proteins to components bound at the 3′ splice site. SR proteins have been shown to promote binding of U1 snRNP to 5′ splice sites (Eperon et al., 1993; Kohtz et al., 1994; Zahler & Roth, 1995). In exon definition, U1 stimulates splicing of major or minor upstream introns by base pairing to the downstream 5′ splice site and interacting—presumably indirectly—with U2 or U12 snRNPs across the exon. SR proteins bound to purine-rich enhancers may also recruit U1 snRNP (reviewed in Valcárcel et al., 1995; Manley & Tacke, 1996; Cáceres & Krainer, 1997). However, whether U1 plays a role in the recognition or function of splicing enhancers remains controversial. Mutational analysis and competition experiments did not support a mechanism involving direct base pairing between the IgM enhancer and U1 snRNA, suggesting that U1 may only have an indirect general stimulatory effect on enhancer-mediated splicing (Tanaka et al., 1994). Other experiments suggested that U1 is not required for the function of a purine-rich enhancer in ASLV (Achsel & Shimura, 1996). It has also been shown that the U1 5′ terminus is not required for the enhancer-mediated stimulation of U2AF binding to the upstream polypyrimidine tract (Wang et al., 1995).

Here we present functional evidence that purine-rich enhancers stimulate AT-AC splicing independently of U1 snRNP, suggesting that there is no intrinsic requirement for U1 in enhancer function in either the major or minor pathway. First, cleavage of the 5′ terminus of U1 snRNA by RNase H does not inhibit enhancer function (Fig. 2). Second, to address the possibility that U1 participates in enhancer function other than by base pairing interactions mediated by its 5′ end, U1 snRNP was inactivated by binding of a complementary 2′-*O*-methyl oligonucleotide. Previous studies showed that U1 snRNA lacking the 5′ terminus can still function in splicing of spliced leader and adenovirus major late chimeric pre-mRNAs (Bruzik & Steitz, 1990). This particular function of U1 is resistant to RNase H-mediated inactivation but is sensitive to treatment with a 2′-*O*-methyl oligonucleotide (Seiwert & Steitz, 1993). In contrast, we found that treatment with this oligonucleotide does not inhibit SCN4AENH1 AT-AC splicing (Fig. 3). Finally, we depleted U1 snRNP from nuclear extract by affinity chromatography with an antisense oligonucleotide, and found that exonic enhancers remained functional in the U1-depleted extract (Fig. 4). Because this treatment severely compromised β-globin splicing, exon definition, and sodium channel pre-mRNA splicing via conventional cryptic splice sites, it seems unlikely that the trace amounts of remaining U1 are sufficient to allow efficient U1-dependent enhancer function. The depletion method probably does not remove U1 particles devoid of the U1 5′ terminus (U1*). U1* is thought to arise in vitro (Lerner et al., 1980) and is not functional in spliceosome assembly (D.A. Wassarman & Steitz, 1993). It is difficult to rule out the possibility that trace amounts of U1* participate in enhancer function. However, it was recently reported that SR proteins promote binding of U1* snRNA to an enhancer-containing RNA much less efficiently than they do in the case of intact U1 snRNA (Stark et al., 1998). Taken together, the results of U1 cleavage, debilitation, and depletion strongly suggest that purine-rich enhancers function independently of U1 snRNP.

The previously noted binding of U1 snRNP at or near certain enhancer elements may be fortuitous or per-

haps related to a process other than splicing enhancement. We note that both the IgM and ASLV enhancers, for which U1 binding under splicing conditions was reported, are located in 3′-terminal exons. Therefore, we favor the idea that U1 binding at 3′ terminal exons reflects the interplay between 3′-end processing and splicing of the last intron, that is, definition of the last exon (K.M. Wassarman & Steitz, 1993; Furth et al., 1994; Lou et al., 1996). It has also been proposed that U1 binding to the last exon may play a role in mRNA transport (K.M. Wassarman & Steitz, 1993). It remains formally possible that U1 plays a role in splicing enhancement in the context of the conventional pathway, or that it is required for the function of some enhancers but not others, depending on the sequence context.

Implications for splicing mechanisms

We have demonstrated that heterologous purine-rich enhancers can promote sodium channel AT-AC pre-mRNA splicing in vitro. We described the existence of natural purine-rich sequences, some of which may function as splicing enhancers, in the exons downstream of several AT-AC introns. We further showed that exon definition and enhancer function have different U1 snRNP requirements. In exon definition, the downstream 5′ splice site apparently communicates with components bound at the upstream 3′ splice site, by means of base pairing with U1 snRNA and bridging via SR proteins. In the case of enhancers, SR proteins can directly bind to the enhancers and promote assembly of components at the 3′ splice site. In the major pathway, SR proteins are thought to stabilize the binding of U2AF to the polypyrimidine tract of the upstream 3′ splice site by contacting U2AF^{35}; U2AF^{65} in turn promotes binding of U2 snRNA to the adjacent branch site. Whether AT-AC splicing requires one or both subunits of U2AF, or whether it utilizes an analogous but distinct component, is not known. However, this presumptive component is expected to interact with complexes formed at exonic enhancers and/or at downstream conventional 5′ splice sites. We have been unable to reconstitute AT-AC splicing by complementation of U2AF-depleted extracts with active U2AF^{65}, apparently because a component(s) specific for AT-AC splicing is inactivated by the depletion procedure (data not shown); therefore, whether or not U2AF^{65} itself is involved in AT-AC splicing remains an open question.

Considering the obvious differences in the 3′ splice site elements and sequence context between AT-AC and conventional introns, it is remarkable that the same enhancers can function in AT-AC splicing as well. Given the absence of a polypyrimidine tract, the lack of a requirement for U2 snRNA, and the short distance between the branch and the 3′ splice site, it is conceivable that SR proteins instead contact a component of the U11/U12 di-snRNP to facilitate U12 snRNA binding to the branch site. Further insights into AT-AC pre-mRNA splicing mechanisms await the identification of snRNP and non-snRNP protein components of this pathway.

MATERIALS AND METHODS

Plasmid constructions

A fragment containing exon 2, intron 2, and exon 3 of SCN4A was amplified from the pSP64-SCN4A plasmid DNA using a forward primer with a *Hin*d III site and a reverse primer with an *Eco*R I site and the SCN4AENH1 enhancer sequence (Fig. 1B). This fragment was digested with *Hin*d III and *Eco*R I and subcloned into the corresponding sites of pSP64 (Promega) to generate the pSP64-SCN4AENH12 plasmid. A similar fragment was amplified using the same forward primer and a different reverse primer with a *Bam*H I site and the SCN4AENH3 enhancer sequence (Fig. 1B). This fragment was digested with *Hin*d III and *Bam*H I and subcloned into pSP64 to generate the pSP64-SCN4AENH3 plasmid. Both constructs were confirmed by sequence analysis. pSP64-SCN4AENH12 was linearized with *Eco*R I or *Bam*H I and pSP64ENH3 was linearized with *Bam*H I for use as templates for in vitro transcription with SP6 RNA polymerase to generate pre-mRNA splicing substrates SCN4AENH1, SCN4AENH2, and SCN4AENH3, respectively. The transcripts contain short extensions at both ends, derived from the restriction site or vector. The pSP64-SCN4A, pSP64-SCN4AM, and pSP64-SCN4AS plasmids have been described (Wu & Krainer, 1996, 1997).

In vitro splicing assays and inhibition experiments

Nuclear extract and substrate preparation, and conditions for in vitro splicing in Figures 1 and 2 were as described (Wu & Krainer, 1996), with the incubation times indicated in the figures or legends. Slight changes in reaction conditions result in optimal AT-AC splicing with different extract batches. In Figures 3 and 4, we used 5.5 mM $MgCl_2$, 2.5 mM ATP, and 40 mM creatine phosphate, which resulted in more efficient basal AT-AC splicing than reported previously. Oligonucleotide-directed RNase H cleavage experiments and the oligonucleotides sequences were as described (Wu & Krainer, 1997). 2′-*O*-methyl oligonucleotide inhibition experiments were performed as described (Wu & Krainer, 1997) using a 2′-*O*-methyl oligonucleotide (Oligos Etc., Inc.) complementary to U1 snRNA position 1–14. All experiments were performed at least twice with similar results.

U1 snRNP depletion and northern blotting

U1 snRNP depletion was performed by modification of a published protocol (Barabino et al., 1990), using a biotinylated 2′-*O*-methyl oligonucleotide complementary to U1 snRNA position 1–14 followed at the 3′ end with 4 biotin-dU residues. Splicing extract was used instead of undialyzed nuclear lysate, NP-40 was omitted, and the oligonucleotide concentration was 7.5 μM. Nuclear extract was incubated for

30 min at 30 °C with the biotinylated oligonucleotide and added to an equal volume of streptavidin-agarose beads (Sigma) that had been pre-blocked as described (Barabino et al., 1990) and washed with extract dialysis buffer. The suspension was rocked in the cold room for 45 min and the supernatant was incubated again with an equal volume of fresh streptavidin-agarose beads. For mock depletion, the extract was treated in the same manner, except that the oligonucleotide was omitted. Further batch treatment with streptavidin-agarose to achieve more extensive U1 depletion resulted in nonspecific inhibition of splicing (data not shown). RNA was extracted and analyzed by Northern blotting with U1- and U2-specific RNA probes (Konarska & Sharp, 1987) transcribed from plasmids kindly provided by M. Konarska.

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