U2 snRNA sequences that bind U2-specific proteins are dispensable for the function of U2 snRNP in splicing

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Previously we showed that microinjection of purified U2 snRNA from HeLa cells into *Xenopus laevis* oocytes, depleted of their endogenous U2 snRNPs by oligonucleotide-targeted degradation, led to assembly of hybrid snRNPs that were fully functional for splicing of SV40 late pre-mRNA. We have extended these results by examining features of U2 RNA that are required for its role in splicing. Injection of *Xenopus* U2 snRNA transcribed in vitro by T7 RNA polymerase, differing in sequence from authentic U2 by only one nucleotide, although capable of efficient assembly into snRNP-like particles, did not complement U2-predepleted oocytes for splicing. However, when injected into pretargeted oocytes, a plasmid containing *Xenopus* U2 snRNA sequences resulted in synthesis of U2 snRNA that was assembled into snRNPs capable of mediating splicing of SV40 late pre-mRNA. This allowed us to test several U2 RNA mutants for their function in splicing. Mutants with sequences deleted within U2 stem-loops I and II, although efficiently assembled into snRNP-like particles upon injection, failed to restore splicing. Interestingly, however, injection of a mutant that lacks the binding site for the U2-specific proteins A' and B'', restored pre-mRNA splicing. These data suggest that the direct binding of U2-specific proteins with snRNA is not essential for the function of U2 snRNPs in splicing of pre-mRNA.

[Key Words: U2-specific proteins; snRNP; splicing; SV40 RNA; *Xenopus* oocytes]

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The U2 small nuclear ribonucleoprotein (snRNP) is one of the most abundant nucleoplasmic snRNPs in higher eukaryotes. It consists of one small nuclear RNA (snRNA) and nine associated proteins (for review, see Guthrie and Patterson 1988; Luhrmann 1988). Of these proteins, seven structural polypeptides, designated B', B, D, D', E, F, and G, are common to all of the Sm class of snRNPs (for review, see Luhrmann 1988). At least one of these polypeptides contains the epitope that is recognized by autoimmune sera with Sm specificity (Hinterberger et al. 1983; Kinlaw et al. 1983; Luhrmann 1988). By mutational analysis, it has been shown that these polypeptides are associated with a highly conserved sequence (AU_nG, *n* = 4–6) within U2 snRNA, termed the Sm-binding site (Mattaj and De Robertis 1985; Mattaj et al. 1986). It has also been demonstrated that there are two U2-specific proteins, A' and B'', that require the two 3' loops of U2 snRNA for binding (Mattaj and De Robertis 1985).

Addition of oligonucleotides complementary to corresponding regions within U2 snRNA to extracts prepared from mammalian cells (Black et al. 1985; Krainer and Maniatis 1985) or yeast cultures (Ruby and Abelson 1988), as well as injection into the nuclei of *Xenopus laevis* oocytes (Pan and Prives 1988; Pan et al. 1989), results in specific degradation of U2 snRNA and dramati-

cally inhibits splicing of pre-messenger RNA (pre-mRNA). The U2 snRNP, which binds to the branch site region of pre-mRNA (Black et al. 1985; Ruskin et al. 1988) in a reaction involving a Watson–Crick base-pairing interaction (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989), is required for the formation of the functional splicing complex, termed the spliceosome (for review, see Steitz et al. 1988).

Although snRNA–protein interactions within the U2 snRNP have been well studied, little is known of the function of U2 snRNP proteins. Efficient reconstitution systems would be valuable in understanding their roles. Several reports have been made recently that describe attempts to develop in vitro assembly systems using synthetic snRNAs (Hamm et al. 1987; Patton et al. 1987; Kleinschmidt et al. 1989; Pikielny et al. 1989). Although snRNP-like particles can be formed in cell-free extracts, their function in splicing has not been addressed directly. The *X. laevis* oocyte serves as an excellent alternative for studies of snRNP function for several reasons. The endogenous U1 or U2 snRNPs can be destroyed by oligonucleotide-targeted degradation, and the oligonucleotides, themselves, are also degraded rapidly (Pan and Prives 1988). There is little or no endogenous snRNA transcription in mature oocytes (Newport and Kirschner 1982); thus, the oligonucleotide-injected oo-

cyte remains depleted of the corresponding snRNA. Furthermore, the oocyte stockpiles snRNP-associated proteins in its cytoplasm that are capable of binding to exogenously introduced snRNAs, forming snRNP-like particles, which then move to the nucleus where splicing occurs (De Robertis et al. 1982; Forbes et al. 1983; Zeller et al. 1983; Fritz et al. 1984; Riedel et al. 1987; Tollervey and Mattaj 1987). Finally, the mature oocyte is capable of correctly splicing several pre-mRNAs (Green et al. 1983; Wickens and Gurdon 1983; Fradin et al. 1984; Manley et al. 1986).

Recently we reported that purified U2 snRNAs from HeLa cells, when injected into *Xenopus* oocytes that had been depleted of their endogenous U2 snRNPs, are assembled into snRNPs that are fully functional for splicing of SV40 late pre-mRNA (Pan and Prives 1988). Having developed this complementation system, now we have performed experiments to determine the requirements for assembly of functional U2 snRNPs. We synthesized large quantities of U2 snRNAs in vitro and analyzed their function in mediating splicing, and we also examined the abilities of several U2 mutants constructed previously (Mattaj and De Robertis 1985; Mattaj 1986), some of which abolish the binding of U2 snRNA to either Sm proteins or U2-specific proteins, to complement U2-depleted oocytes for splicing of SV40 late pre-mRNA.

Results

Construction of templates for synthetic U2 RNAs

Two templates were engineered to synthesize U2 RNA in vitro by T7 RNA polymerase (Fig. 1A). The template for T7U2⁺, a gift from J. Hamm and I. Mattaj, was constructed as described by Hamm et al. (1987) (for details, see Materials and methods). Compared to the authentic U2 (Reddy and Busch 1988), the synthetic T7U2⁺ con-

tains three additional G residues at the 5' end and one extra U nucleotide at the 3' end. To construct a U2 RNA (T7U2⁺) that differs from authentic U2 by only one additional G residue at the 5' end, we used the polymerase chain reaction (PCR). For this purpose, two oligonucleotide primers were synthesized. The 5' primer (27 nucleotides) contained sequences within the T7 promoter (Dunn and Studier 1983), one G residue, and nine nucleotides of the 5' end of U2. The 3' primer (15 nucleotides) consisted of sequences of the precise 3' end of U2. The two primers were hybridized to the T7U2⁺ template and subjected to PCR amplification (Saiki et al. 1988). Then the PCR products were purified and used as templates for in vitro transcription.

The U2 RNA made from the in vitro transcription reaction was analyzed by 8% polyacrylamide-urea gel electrophoresis and stained with ethidium bromide (Fig. 1B). Using similar reaction conditions, we observed that transcription of T7U2⁺ RNA was markedly more efficient than that of T7U2⁺ (cf. lanes d and f). This is consistent with a previous report indicating that three G residues from the T7 promoter are required to obtain efficient initiation (Hamm et al. 1987). Nevertheless, sufficient amounts of T7U2⁺ were obtained for further analysis in oocytes.

Injected synthetic U2 RNA is efficiently assembled in oocytes

Previous experiments have demonstrated that U2 snRNA synthesized from an injected plasmid template in oocytes is efficiently assembled with Sm proteins and trimethylated at the cap site (Mattaj 1986). This was confirmed, as shown in Figure 2A. We then similarly analyzed the in vitro-transcribed T7U2⁺ RNAs labeled with [α -³²P]GTP after microinjection into the cytoplasm of oocytes. The injected oocytes were incubated for either 0 min or 24 hr before extraction. Three separate

Figure 1. In vitro transcription of U2 snRNAs. (A) Diagram indicates partial sequence of the templates (for construction, see Materials and methods), as well as their transcripts synthesized by T7 RNA polymerase in vitro. Vertical arrows show where authentic U2 transcript (188 nucleotides) initiates (+1) and terminates (+188). The additional nucleotides introduced by template construction are underlined. (B) Synthetic U2 snRNAs T7U2⁺ and T7U2⁺ were transcribed by T7 RNA polymerase and analyzed on 8% polyacrylamide-urea gels; (lanes b and e) 0.1 μ g of purified HeLa cell U2 snRNA and 1.3 μ g of total nuclear RNA isolated from $\sim 10^6$ HeLa cells, respectively; (lanes c and f) 0.1 and 0.5 μ g of T7U2⁺ RNA, respectively; (lane d) 50 ng of T7U2⁺ RNA. Lanes a and g contain pBR322 DNA fragments digested by *Msp*I.

Figure 2. Properties of synthetic U2 RNA in oocytes. (A) Oocytes in groups of 10, injected with 10 ng of XU2 plasmid and 10 nCi of [α - 32 P]GTP were extracted after a 24-hr incubation. (Lane a) Total RNA synthesized; (lane b) oocyte extracts immunoprecipitated with anti-Sm antibodies; (lane c) purified RNA after immunoprecipitation with anti-TMG antibodies. (B) In vitro-synthesized T7U2⁺4 uniformly labeled with [α - 32 P]GTP was injected into cytoplasm of oocytes in groups of 10. After 0 min (lanes b, d, and f) or 24 hr (lanes c, e, and g) of incubation, total RNA isolated was either run directly on 8% gels (lanes b and c) or immunoprecipitated with anti-TMG antibodies (lanes f and g). Extracts of oocytes were immunoprecipitated with anti-Sm antibodies (lanes d and e). (C) Oocytes that had been injected with T7U2⁺4, 24 hr earlier, received 20 ng of the U2a oligonucleotide as a second injection. After a 1-hr (lanes a and c) or 2-hr (lanes b and d) incubation, total RNA extracted from one oocyte was either run directly on 8% gels (lanes a and b) or immunoprecipitated with anti-TMG antibodies (lanes c and d). Lanes a (in A and B) and e (in C) contain 32 P-labeled pBR322 DNA fragments digested with *MspI*.

analyses were performed on these two groups of oocytes (Fig. 2B). First, RNAs extracted from these oocytes were directly analyzed on 8% polyacrylamide-urea gels. The levels of T7U2⁺4 RNA detected from these two groups of oocytes were similar, indicating that the injected synthetic U2 RNA was very stable. Second, extracts made from these two groups were incubated with anti-Sm antibodies. Only the 24-hr sample group was efficiently immunoprecipitated with anti-Sm antibodies, indicating that the injected T7U2⁺4 RNA was efficiently assembled with Sm proteins. Third, RNA samples were immunoprecipitated with anti-trimethylguanosine (TMG) antibodies. Here, too, only the 24-hr oocyte group reacted with the anti-TMG antibodies, suggesting that the cap of the injected T7U2⁺4 RNA was efficiently trimethylated. It should be noted that we also determined that the majority of the injected synthetic T7U2 RNA was found in the oocyte nucleus after 24 hr of incubation (data not shown).

To test whether the 5' end of the injected T7U2⁺4 RNA was accessible to oligonucleotides in oocytes, as has been shown previously for either authentic HeLa cell or amphibian U2 snRNA (Black et al. 1985; Pan et al. 1989), a 15-mer (U2a), complementary to 15 nucleotides at the 5' end of U2 snRNA (for sequence, see Materials and methods), was introduced into oocytes that had been injected with T7U2⁺4 RNA 24 hr previously. After incubation for an additional 1 or 2 hr, RNA from the oocytes was extracted. The RNA samples were either directly subjected to gel electrophoresis or immunoprecipitated with anti-TMG antibodies (Fig. 2C). The U2a treatment resulted in accumulation of a shorter U2 species that was not recognized by anti-TMG antibodies. Thus, the 5' end of the injected T7U2⁺4 RNA is acces-

sible for hybridization with the oligonucleotide like its authentic counterpart. Similar data were obtained when the in vitro-synthesized T7U2⁺1 was examined for stability and assembly in oocytes (data not shown). Therefore, the in vitro-synthesized U2 RNAs were assembled into snRNP particles that resembled the authentic U2 counterpart in Sm or TMG reactivity, as well as their availability to the 5' end-specific oligonucleotide.

Synthetic U2 RNAs do not restore splicing of SV40 late pre-mRNA in U2-predepleted oocytes

The function of the synthetic U2 RNAs was analyzed by the complementation system established earlier (Pan and Prives 1988). This involved three injections: (1) A 15-nucleotide oligomer (U2b) complementary to the second stem-loop of U2 snRNA, was introduced intranuclearly to induce oligonucleotide-directed cleavage of the endogenous U2 snRNA; (2) after 4 hr of incubation, purified HeLa cell U2 snRNA or in vitro-transcribed T7U2 RNA was injected to generate new U2 snRNP-like particles; and (3) 16 hr later, SV40 DNA was injected into the nucleus, resulting in the synthesis of viral late RNA precursors. As has been demonstrated previously [Miller et al. 1982; Wickens and Gurdon 1983; Fradin et al. 1984], when SV40 DNA (form I) was injected, large quantities of viral late RNA were synthesized and spliced primarily into the 19S form shown by S1 nuclease mapping analysis (Fig. 3A). In contrast to SV40-infected monkey cells, little or no 16S spliced RNA was made in oocytes (cf. lanes a and b). Confirming our previous observation (Pan and Prives 1988), the production of spliced 19S RNA was abolished in oocytes that had been injected with the U2b oligonucleotide (lane c) with

Figure 3. Synthetic T7U2 RNAs do not form functional snRNPs in oocytes. (A) Groups of 10 oocytes were injected with 20 ng of the U2b oligonucleotide (lanes c–h) for 4 hr, and then received a second injection of 1 ng of purified HeLa cell U2 snRNA (lane d), 1 or 2 ng of T7U2⁺ (lane e or f), and 1 or 2 ng of T7U2⁺ (lane g or h). Sixteen hours later, 2.5 ng of SV40 DNA was injected intranuclearly (lanes b–h) and incubated for another 16 hr. Purified RNAs were analyzed by S1 nuclease mapping (Materials and methods). Lane a contains similarly treated RNA from SV40-infected CV-1 cells. Note that larger amounts of spliced 16S than 19S RNA species are produced in monkey cells. (B) Map indicates the expected S1-resistant fragments corresponding to spliced and unspliced viral late RNAs.

the concurrent accumulation of greatly increased quantities of unspliced RNA. On cytoplasmic injection of the purified U2 snRNA from HeLa cells, splicing to form 19S RNA was restored (lane d). However, when either T7U2⁺ or T7U2⁺ RNA was injected into U2-predepleted oocytes in quantities similar to HeLa cell U2 RNA, 19S RNA splicing was not detected (lanes e–h). Even when several concentrations of T7U2 RNAs, ranging from 0.5 to 4 ng, were microinjected, splicing was never restored (data not shown). Intranuclear injection of the T7-synthesized U2 RNAs also failed to restore splicing (data not shown). Thus, microinjection of in vitro-synthesized U2 snRNAs, even one differing by only one nucleotide from authentic U2 snRNA, although capable of assembly into trimethyl-capped snRNP-like particles, failed to complement U2-predepleted oocytes for splicing of SV40 late pre-mRNA. Possible reasons for this are discussed later.

Injection of a plasmid expressing U2 snRNA restores splicing in U2-predepleted oocytes

As described previously (Mattaj and De Robertis 1985) and confirmed in this study (see Fig. 2A), injection of a plasmid containing the *Xenopus* U2 gene (XU2) led to synthesis of U2 snRNA that was efficiently assembled into U2 snRNP-like particles in oocytes. To determine the function of these particles in splicing, two sequen-

tial injections were performed: First, we coinjected SV40 DNA and the U2b oligonucleotide, which resulted in depletion of the endogenous U2 snRNPs and accumulation of unspliced viral late RNA precursors (Pan and Prives 1988; Pan et al. 1989); after a 16-hr incubation, we introduced the XU2 plasmid into oocytes, resulting in the assembly of new U2-like particles. To test whether such manipulations affect the ability of oocytes to synthesize and assemble U2 snRNPs, XU2 plasmid and [α -³²P]GTP were coinjected into oocytes that had or had not been injected with SV40 DNA and the U2b oligonucleotide. RNA purified from these two groups of oocytes was directly analyzed on 8% polyacrylamide–urea gels, and extracts made from them were immunoprecipitated with anti-Sm antibodies (Fig. 4A). Because the quantities of RNAs synthesized and Sm-immunoprecipitated in the singly or doubly injected oocytes were essentially identical, we concluded that the oocytes were fully capable of assembling U2 snRNPs under the experimental conditions necessary to observe complementation. S1 mapping analysis was then performed on these samples (Fig. 4B), and oocytes that had been injected with SV40 DNA and the U2b oligonucleotide and that had received either nothing or pBR322 DNA were found to be incapable of splicing SV40 late pre-mRNA (lanes b and c). Oocytes that had received the XU2 plasmid as their second injection, however, accumulated spliced 19S RNA in amounts that were equivalent to those made in

Figure 4. An injected wild-type U2 plasmid generates functional snRNPs in oocytes. (A) Two and one-half nanograms of SV40 DNA and 20 ng of the U2b oligonucleotide were injected into nuclei of oocytes in groups of 10 (lanes c and f). Twenty-four hours later, 10 ng of XU2, along with [α - 32 P]GTP, was injected (lanes b, c, e, and f) and incubated for an additional 16 hr before extraction. Total RNAs were analyzed directly by gel electrophoresis (lanes b and c). Oocyte extracts were immunoprecipitated with anti-Sm antibodies (lanes e and f). Lanes a and d are *MspI*-digested pBR322 DNA fragments. (B) Oocytes in groups of 10 were injected first with SV40 DNA (lanes a–d), in the presence (lanes b–d) or absence (lane a) of 20 ng of the U2b oligonucleotide. Sixteen hours later, the oocytes were injected with 10 ng of either pBR322 DNA (lane c) or XU2 (lane d) and incubated for additional 16 hr prior to extraction. RNA from these oocytes was similarly analyzed by S1 nuclease mapping as in Fig. 3.

oocytes injected with SV40 DNA alone (cf. lanes a and d). Thus, injection of a plasmid encoding *Xenopus* U2 snRNA is capable of restoring SV40 late RNA splicing in U2-predepleted oocytes.

Assembly of U2 mutants in U2-predepleted oocytes

The observation that a microinjected plasmid expressing *Xenopus* U2 snRNA complemented U2-predepleted oocytes for splicing SV40 late pre-mRNA prompted us to analyze the function of several U2 mutants ΔA – ΔF , as well as 323 and 331 (see Fig. 5A; Mattaj and De Robertis 1985; Mattaj 1986). Previous experiments characterizing these DNA mutants in oocytes have demonstrated that (1) mutants ΔA and ΔB containing altered sequences within U2 stem–loops I and II are efficiently transcribed and assembled with Sm as well as U2-specific proteins (A' and B'); (2) whereas transcripts made from mutants ΔD – ΔF containing altered sequences within U2 stem–

loops III and IV are somewhat unstable, they are still capable of associating with Sm proteins but are not immunoprecipitated by antibodies specific for the A' and B' proteins, (3) a mutant that removes the Sm-binding site (ΔC) is not precipitated by antibodies against any U2 snRNP proteins, whereas reinsertion of the Sm-binding domain to other regions of U2 snRNA, creating mutants 323 and 331, partially restores its binding capability; and (4) all mutants except ΔC , 323, and 331 accumulate efficiently in the oocyte nucleus, although 323 and 331 do enter the nucleus. To ensure that these mutants can be assembled in pretargeted oocytes, each of the constructs ΔA – ΔF , 323, and 331 (kindly provided by I.W. Mattaj) was injected along with [α - 32 P]GTP into oocytes that had received SV40 DNA and the U2b oligonucleotide 16 hr earlier. Oocytes were extracted and immunoprecipitated with anti-Sm antibodies 24 hr later (Fig. 5B). The results were essentially consistent with the experiments described previously (Mattaj and De Robertis 1985; Mattaj

Figure 5. Assembly of mutant U2 snRNPs in U2-predepleted oocytes. (A) Secondary structure of *Xenopus* U2 snRNA is drawn according to Keller and Noon (1985). Sequence changes made in the eight U2 mutants used in this study are shown below, based on previous publications (Mattaj and De Robertis 1985; Mattaj 1986). (B) Ten nanograms of each of the U2 mutant plasmids or of the wild-type XU2 plasmid were injected along with [α - 32 P]GTP into oocytes that had been injected previously with 2.5 ng of SV40 DNA and 20 ng of the U2b oligonucleotide 16 hr earlier. After incubation for another 24 hr, the oocytes were extracted and immunoprecipitated with anti-Sm antibodies prior to purification of RNA. The major precipitated U2 RNAs are indicated by arrowheads.

1986), although we found that the ΔF mutant assembled with Sm antigens far more efficiently than reported previously and assembly of mutants 323 and 331 was observed to be even less efficient than had been described. This may not be surprising because previous studies showed that differences in concentration of injected DNA within batches of oocytes significantly affect the abundance of mutant transcripts synthesized (Mattaj and De Robertis 1985).

U2 mutants define regions required for splicing of SV40 late pre-mRNA in oocytes

The function of U2 mutants in splicing was tested as described above (Fig. 4). ΔA – ΔF , 323, and 331 plasmids were each injected into oocytes that had been injected previously with SV40 DNA and the U2b oligonucleotide 16 hr earlier. After an additional 24 hr of incubation, RNA from oocytes was extracted and subjected to S1 nuclease mapping (Fig. 6A). In contrast to wild-type XU2, mutants ΔA and ΔB did not restore splicing in U2-predepleted oocytes. As expected, injection of ΔC , the mutant lacking the Sm-binding domain, failed to com-

plement oocytes for splicing as well. With mutants ΔD , ΔE , 323, and 331, the viral late RNAs were also mostly unspliced. However, the inability of these mutants to complement splicing may have been the result of their inefficient assembly into snRNP-like particles, as determined by Sm immunoprecipitations (see Fig. 5B), although other possibilities cannot be ruled out at this stage. In contrast to all other mutants tested, when the U2-predepleted oocytes received the ΔF U2 plasmid as their second injection, significant amounts of spliced 19S RNA were produced. In this experiment, restoration of splicing with ΔF was estimated to be ~50% efficient, as judged by the observed ratio between 19S RNA and the unspliced viral late RNA precursor. From a number of additional complementation experiments that we performed, XU2 was frequently found to be more efficient than ΔF in restoring splicing in pretargeted oocytes. However, in other experiments, ΔF restored splicing more completely (see Fig. 6B). The other mutants were also tested over a wide range of concentrations; however, no complementation for splicing was observed in any case (data not shown). Thus, the mutant ΔF , which lacks the binding sites for U2-specific pro-

Figure 6. Mutant ΔF U2 RNA restores splicing in U2-predepleted oocytes. (A) Groups of 10 oocytes were first injected with 2.5 ng of SV40 DNA and 20 ng of the U2b oligonucleotide and incubated for 16 hr (lanes a–j). Then the oocytes were injected with 10 ng of each of the following U2 constructs: XU2 (lane b), ΔA (lane c), ΔB (lane d), ΔC (lane e), ΔD (lane f), ΔE (lane g), ΔF (lane h), 323 (lane i) and 331 (lane j). Twenty-four hours later, oocytes were extracted and analyzed by S1 nuclease mapping, as in Fig. 3. (B) Oocytes that had been injected previously with SV40 DNA and U2b (lanes a–d), received as their second injection, 10 ng of XU2 (lane b), or 2.5 ng (lane c) or 20 ng (lane d) of mutant ΔF plasmid. RNA from injected oocytes was then purified and analyzed by S1 nuclease mapping.

teins (A' and B''), still maintained the ability to mediate splicing of SV40 late pre-mRNA. Because the efficiency of mutant ΔF was often somewhat lower, it is possible that the A' and B'' U2-specific proteins are necessary to confer maximal U2 splicing function.

The presence of mutants ΔA and ΔB does not inhibit the function of wild-type U2

Because mutant ΔA or ΔB was efficiently assembled into snRNPs but was nonfunctional in splicing of SV40 late pre-mRNA, it was possible that ΔA or ΔB associated with other splicing elements but generated spliceosome-like complexes that were defective in splicing. If this were the case, these mutants should compete with the wild-type U2 snRNPs. To test this, each of the mutants, ΔA – ΔC , was coinjected with XU2 into the pretargeted oocytes. As shown in Figure 7A, both mutant and wild-type U2 snRNAs were expressed from each pair of constructs injected, and with the exception of ΔC , all associated with Sm antigens. However, all sets of coinjections fully restored the ability of the U2-predepleted oocytes to splice SV40 late pre-mRNA (Fig. 7B). Thus, mutants ΔA and ΔB , although generating equivalent quantities of snRNP particles, as determined by Sm antibody reactivity, did not block the ability of wild-type U2 snRNPs to mediate SV40 late pre-mRNA splicing.

Discussion

We used our previous observation, that purified HeLa cell U2 snRNA can form a functional human–amphibian hybrid snRNP in *X. laevis* oocytes, to explore

further properties of U2 snRNA that contribute to its role in splicing. We found that a plasmid encoding *Xenopus* U2 snRNA, when injected into U2-predepleted oocytes, restored splicing of SV40 late pre-mRNA. Unexpectedly, however, *Xenopus* U2 RNA synthesized in vitro by T7 RNA polymerase failed to complement splicing-deficient oocytes. Two major differences exist between the in vitro-transcribed U2 RNAs and those synthesized in oocytes. First, the synthetic U2 RNAs contained additional residues: three extra 5' G nucleotides and one 3' U residue for T7U2⁺, and only one additional G nucleotide at the 5' end for T7U2⁺. Second, being synthesized in the in vitro reaction by T7 RNA polymerase, the synthetic U2 RNAs, at least prior to injection into oocytes, lacked modified nucleotides. Authentic U2 snRNAs, including those from human cells, are known to contain a proportion of post-transcriptionally modified nucleotides greater than that of other snRNAs (Reddy and Busch 1988). It is of particular interest that these modified nucleotides are localized exclusively in the 5' half of U2 (Reddy and Busch 1988) in the region containing sequences required for splicing, including the branch site-binding domain. Furthermore, some of these modifications, at least in the case of *Xenopus* U2 snRNA, are developmentally controlled (Lund and Dahlberg 1987). Although not documented specifically for U2 RNA, there are reports that *Xenopus* oocytes exhibit levels of modification activities somewhat lower than those of *Xenopus* kidney cells in culture (Kressmann et al. 1978). Therefore, it is conceivable that at least some modifications crucial for the function of U2 are suboptimal in oocytes. However, because we showed that an injected plasmid encoding *Xenopus* U2 did give rise to functional U2 snRNPs, it is clear that if

Figure 7. Mutants ΔA , ΔB , and ΔC do not compete for splicing with wild-type U2 RNA. (A) Ten nanograms of either mutant ΔA (lanes *b* and *e*), ΔB (lanes *c* and *f*), or ΔC (lanes *d* and *g*) was injected, along with $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ and 10 ng of XU2 into oocytes in groups of 10. Total RNAs from oocytes after 24 hr of incubation were analyzed by direct gel electrophoresis (lanes *b–d*). Oocyte extracts were immunoprecipitated with anti-Sm antibodies (lanes *e–g*). The mutant U2 RNAs synthesized or immunoprecipitated are marked by arrows. (B) Groups of 10 oocytes that had been injected with SV40 DNA (2.5 ng) and the U2b oligonucleotide (20 ng) and incubated for 24 hr (lanes *a–d*), were coinjected with 10 ng of XU2 and 10 ng of each of the following mutants: ΔA (lane *b*), ΔB (lane *c*), and ΔC (lane *d*). Total RNA from each group was extracted after an additional 16 hr of incubation and subjected to S1 analysis, as described in Fig. 3.

modifications are important for splicing function, they must occur in injected oocytes, to some extent. Although it has been reported that some snRNA modifications take place in the cytoplasm (Eliceiri 1980), it is possible that others may occur in the nucleus, possibly in a manner that is somehow dependent on transcription. Simply being present in the nucleus, however, may not be sufficient because we observed that when the in vitro-transcribed U2 RNAs were injected into the nucleus, they similarly failed to restore splicing in U2-pre-depleted oocytes.

The second possibility, that the presence of an extra 5' G residue renders U2 snRNA inactive in splicing, is considered to be less attractive because this additional nucleotide is not likely to alter the predicted snRNA secondary structure. Although it is feasible to construct a template encoding a perfect U2 RNA by sequence using the PCR method described, the loss of T7 RNA polymerase activity after deletion of yet another G from the promoter is likely to render the quantities of U2 RNA synthesized too low to be useful. However, to determine whether the additional G is deleterious to its function in

splicing, we are currently constructing a mutant *Xenopus* U2 plasmid containing one additional G residue inserted at the 5' terminus. It should be noted that most reported attempts to generate functional reconstituted snRNPs in extracts either from cultured mammalian cells (Patton et al. 1987; Kleinschmidt et al. 1989; Pikielny et al. 1989) or *Xenopus* eggs (Hamm et al. 1987) used in vitro-transcribed U snRNAs. Under the same conditions, U4/U6, but not U2 and U5, have been assembled into splicing complexes (Pikielny et al. 1989). As was shown by Patton et al. (1987), the majority of reconstituted U1 RNA molecules are not modified in vitro. Should the difference between U4/U6 and U2 therefore be the result of the lack of appropriate modification of U2, this would provide an important example of the roles of nucleotide modifications in snRNA function.

The complementation system using U2 plasmids described in this study provides a way to study the function of snRNA sequences. Among the U2 mutants tested, ΔA and ΔB , although assembled into snRNP-like particles with similar efficiency as that of wild-type U2, failed to complement splicing of SV40 late pre-mRNA. The functional importance of the stem-loop I structure of U2 (nucleotides 7–26, Fig. 5A), which is significantly altered by the ΔA mutant, has been examined previously by oligonucleotide-targeted degradation (Frendewey et al. 1987). Removal of the 5' end of U2 snRNA from nucleotide 1–15 results in complete loss of formation of any splicing complexes in nuclear extracts, indicating that this particular region is required for the assembly of the presplicing complex. More is known about the region that ΔB alters, a stretch of single-stranded sequence and part of stem-loop II structure of U2 (nucleotides 39–71). Cleavage of U2 sequence within this region (nucleotides 28–42) blocks formation of the mature spliceosome but not the pre-splicing complex (Frendewey et al. 1987), indicating that this domain is involved in the interaction with other splicing elements at a later stage of the splicing process. We (Pan and Prives 1988) and others (Black et al. 1985) have shown that U2 snRNPs are not stable after cleavage of this region. Thus, this domain of U2 also contributes to the stability, as well as the function, of the U2 snRNPs. Even more important, a consensus sequence within this region (nucleotides 33–39) has been found to base-pair with the branch site of pre-mRNA in yeast (Parker et al. 1987), and, more recently, in mammalian cells (Wu and Manley 1989; Zhuang and Weiner 1989). The observation that neither ΔA nor ΔB inhibited the function of the wild-type U2 in splicing upon coinjection is consistent with the notion that the regions that have been altered in these mutants are required for the direct interactions of U2 snRNPs with other splicing factors forming functional complexes. Although it is impossible to identify such factors at this stage, it is of interest to recall that unlike the wild-type U2, neither mutant ΔA nor mutant ΔB is capable of interacting with U1 snRNPs (Mattaj et al. 1986). To further define the sequences in the U2 stem-loop I and II regions that are involved in their splicing func-

tion, more refined deletion and base-substitution mutagenesis protocols are in progress.

Perhaps our most surprising observation is that the mutant ΔF , lacking the binding site for U2-specific proteins A' and B'' (stem-loop IV), still maintained the ability to mediate splicing. Although these experiments strongly suggest that direct binding of the two U2-specific proteins with snRNA is not essential for the function of U2 in splicing, they do not demonstrate that these proteins are dispensable for such activity. It has been shown that the A' and B'' proteins interact both with the Sm-binding proteins (Mattaj and De Robertis 1985) and directly with RNA in the 3' end of U2 (Fresco et al. 1987). It remains possible that the U2 specific proteins still associate with ΔF via interaction with Sm proteins. Such interaction, however, may be relatively weak and therefore could have escaped detection by immunoprecipitation. Supporting this suggestion is the observation that the A'/B'' proteins, but not the Sm core proteins, can be dissociated from U2 snRNA by high salt concentration (Brunel et al. 1981). Comparison of sequence data has revealed that the human U2-specific protein B'' shares extensive homology with U1-specific protein A (Sillekens et al. 1987). This then suggests that these two proteins are functionally similar. Conceivably, the function of the U1–A protein might substitute for that of U2–B'' polypeptide in ΔF -injected oocytes. Of particular interest is the observation that yeast strains containing substantial deletion in the U2 stem-loop IV region grow significantly more slowly and give rise to U2 RNAs with improperly terminated 3' ends (Shuster and Guthrie 1988). This is consistent with the observation that ΔD , ΔE , and ΔF mutant transcripts, containing alterations in U2 stem-loops III and IV, have short 3' extensions (Mattaj and De Robertis 1985). Thus, the binding of U2-specific proteins to RNA appears to be required for a 3'-processing event but not essential for splicing.

Recently, Hamm et al. (1989) used a similar complementation system to analyze effects of several U2 mutants on splicing of the first intron of adenovirus major late (AdML) transcript. The authors similarly observed that a U2 mutant lacking the stem-loop IV is functional in splicing. However, in contrast to data reported in our study, they showed that the mutant ΔA functions in splicing as well. Previous studies show that different mRNAs differ in their requirement for snRNPs. Splicing of SV40 early pre-mRNA to form either large T or small t mRNA is unusually insensitive to inactivation of U1 snRNPs by either anti-(U1) antibodies (Fradin et al. 1984), or U1 5' end oligonucleotide-targeted degradation (Pan et al. 1989) in *Xenopus* oocytes. It is possible that the authentic U2 5'-end sequence, which has been deleted in ΔA , is indispensable for U2 function in splicing of SV40 19S RNA but not the first intron of AdML. The other important difference is that Hamm et al. (1989) analyzed splicing of an in vitro-synthesized pre-mRNA, and we examined the viral pre-mRNA transcribed in oocytes. Thus, pre-mRNA synthesized in vitro may have altered modifications, secondary struc-

ture, or association with hnRNP proteins that differ from pre-mRNA transcribed in oocytes. Further experiments are in progress to understand the role of the U2 sequences that have been deleted in the ΔA U2 mutant in SV40 RNA splicing.

Materials and methods

Construction of templates for synthesis of U2 snRNAs

The DNA template for T7U2⁺, a gift from J. Hamm and I. Mattaj, was constructed by the method described by Hamm et al. (1987). An oligonucleotide containing the sequence TAA-TACGACTCACTATAGGG [T7 promoter derived from Dunn and Studier (1983)], was inserted into the U2 snRNA gene in the cap site. A DNA sequence containing TAAA was inserted into the U2 snRNA gene in the 3'-termination site to create a *Dra*III site (TTTAAA). To construct the template of T7U2⁺, two oligonucleotides (5' primer, 27 nucleotides, 5'-TAATACGACTCACTATAGATCGCTTCT; 3' primer, 15 nucleotides, 5'-AAGTGCACCGTCCT) were designed and purchased from Operon. The PCR was carried out essentially as described by Saiki et al. (1988). Two primers each at 1 μ M were incubated with 10 ng of T7U2⁺ DNA template in 100- μ l reaction mixtures and subjected to 30 cycles of amplification. The temperatures used for denaturing, annealing, and polymerization were 94°C, 35°C, or 72°C, respectively. The PCR products were gel-purified and sequenced using the Sequenase kit (U.S. Biochemical), which showed that nucleotides 1–153 were identical to U2 genomic sequence (data not shown). However, we were unable to determine the sequence of the last 35 nucleotides at the 3' terminus using three different primers complementary to various regions within the 5'-half of U2.

In vitro transcription of U2 snRNAs

For transcription of capped T7U2⁺, 2 μ g of linearized (*Dra*III) template was incubated with m⁷GpppG (5 mM) in 25- μ l reaction mixtures [Krieg and Melton 1987], either in the presence or absence of 25 μ Ci [α -³²P]GTP (10 μ Ci/ μ l, ICN). After incubation for 30 min at 37°C, the mixtures were treated with DNase I followed by phenol/chloroform-extraction and ethanol-precipitation. The RNAs were normally resuspended in 5 μ l H₂O. For transcription and purification of T7U2⁺ RNA, 40 ng of purified template was used and a similar protocol was followed.

Oligonucleotides

The two oligonucleotides complementary to U2 snRNA used in this study were described previously [Black et al. 1985; Pan and Prives 1988; Pan et al. 1989]. U2a, 5'-AGGCCGA-GAAGCGAT (complementary to U2 at nucleotides 1–15) and U2b, 5'-CAGATACTACACTTG (complementary to U2 at nucleotides 28–42).

U2 DNA plasmids

Wild-type U2 (XU2) and U2 mutants (ΔA – ΔF , 323, and 331) were provided by I.W. Mattaj [Mattaj and De Robertis 1985; Mattaj 1986], some of which (XU2, ΔA – ΔC , and ΔF) were re-subcloned into the pBluescript SK vector (Stratagene).

Microinjection of *X. laevis* oocytes

The methods for preparation and microinjection of oocytes were described previously [Fradin et al. 1984]. Quantities of oli-

gonucleotides, DNAs, or RNAs used for injection were specified in each experiment. Although oligonucleotides and DNAs were injected intranuclearly, RNAs were injected into the cytoplasm. Before being extracted, the injected oocytes were incubated at 19°C for the time period described in the text.

RNA analysis

For gel electrophoresis, total RNAs extracted [Fradin et al. 1984], in amounts equivalent to one-third of an oocyte, were subjected to 8% polyacrylamide–urea gel analysis [Pan and Prives 1988].

For Sm immunoprecipitation, oocytes were extracted according to the method of Mattaj and De Robertis (1985). Extracts of half an oocyte were immunoprecipitated with 5–10 μ l of anti-Sm antibodies [Mattaj and De Robertis 1985].

For TMG immunoprecipitation, purified total RNA equivalent to one oocyte was incubated with 1 μ l of anti-TMG antibody, [a gift from D. Black and J.A. Steitz, using the published procedure of Tollervey and Mattaj (1987)]. RNAs were then purified from these immunoprecipitations and analyzed by 8% polyacrylamide–urea gel electrophoresis.

For S1 nuclease mapping of SV40 late RNA, two alternate probes were prepared: a ³²P-labeled entire SV40 DNA cut with *Bam*HI [Michaeli and Prives 1985; for analyses, see Figs. 3 and 4B], and a fragment of SV40 DNA spanning sites *Eco*RI–*Bgl*II [Fradin et al. 1984; for analyses, see Figs. 6 and 7B]. Either probe was hybridized to RNA isolated from one injected oocyte followed by S1 digestion [Fradin et al. 1984]. The S1-resistant fragments were then analyzed on 1.2% denaturing agarose gels.

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