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Reconstitution of Functional Mammalian U4 Small Nuclear Ribonucleoprotein: Sm Protein Binding Is Not Essential for Splicing In Vitro

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We have developed an in vitro splicing complementation assay to investigate the domain structure of the mammalian U4 small nuclear RNA (snRNA) through mutational analysis. The addition of affinity-purified U4 snRNP or U4 RNA to U4-depleted nuclear extract efficiently restores splicing activity. In the U4-U6 interaction domain of U4 RNA, only stem II was found to be essential for splicing activity; the 5′ loop is important for spliceosome stability. In the central domain, we have identified a U4 RNA sequence element that is important for splicing and spliceosome assembly. Surprisingly, an intact Sm domain is not essential for splicing in vitro. Our data provide evidence that several distinct regions of U4 RNA contribute to snRNP assembly, spliceosome assembly and stability, and splicing activity.

Nuclear pre-mRNA splicing requires the ordered assembly of the splicing substrate into a large ribonucleoprotein (RNP) complex, the spliceosome, containing small nuclear RNPs (U1, U2, U4/U6, and U5 snRNPs) and non-snRNP splicing factors (for recent reviews, see references 6, 28, and 40). The U1 and U2 snRNPs, in conjunction with non-snRNP splicing factors, recognize the 5′ splice site and the branch point region, respectively. Most likely, the U4/U6 and U5 snRNPs interact to form the U4/U5/U6 multi-snRNP before they are incorporated into the spliceosome (4, 25, 37), apparently not through direct pre-mRNA contacts but through snRNP-snRNP interactions (5). The spliceosome proceeds, during its assembly, the two steps of the splicing reaction, and its disassembly, through multiple conformational stages, some of which may be driven by ATP-dependent RNA helicases (for reviews, see references 17 and 36). For example, a major conformational change during splicing destabilizes U4 binding in the spliceosome (8, 11, 26, 35); U6 and U2 RNAs engage in a base-pairing interaction required for the assembly of a stable spliceosome and for splicing (12, 21, 46, 47), and an additional U6-U2 interaction has recently been suggested (32).

On the basis of extensive phylogenetic evidence, a secondary structure model has been proposed for U4 RNA base paired with U6 RNA (18) (for the human U4/U6 RNA hybrid, see Fig. 1). U4 RNA can be divided into three domains, the 5′-terminal U4-U6 interaction domain (stem II, 5′ stem-loop, stem I), the central domain (single-stranded region, central stem-loop), and the 3′-terminal domain (Sm-binding site, 3′ stem-loop). Interestingly, free U4 RNA can also be folded into a conserved secondary structure, in which the 3′-terminal domain remains unchanged compared with the U4/U6 RNA hybrid structure but in which the stem I and stem II regions are partially base paired with each other, thereby extending the 5′ stem-loop structure (33). There are several lines of indirect evidence that U6 RNA sequences play a catalytic role in splicing (10, 14, 42, 43; reviewed in reference 17). Since in the U4/U6 snRNP U6 RNA is stably associated with U4 RNA, U4 has been hypothesized to function as an antisense negative regulator of U6, keeping U6 RNA in an inactive conformation until the unwinding of the U4-U6 base pairing may activate U6 sequences for functioning in splicing (18). Consistent with this hypothesis, U4 RNA has not been conserved as strongly as U6, except for the structural organization of the U4-U6 interaction domain and for the loop sequence of the 5′ stem-loop.

In yeast cells, several proteins are involved in the dynamic interactions between U4, U5, and U6 RNAs. First, PRP4, which requires the 5′ stem-loop region for binding (1, 7, 48), has been implicated in multi-snRNP assembly (9). Second, a U6-binding protein, PRP24, may mediate the formation of the U4-U6 base-pairing interaction (38). Third, on the basis of genetic evidence, the PRP28 protein has been proposed to facilitate U4-U6 unwinding during splicing (41). In higher eucaryotic systems, in contrast, no U4/U6 snRNP-specific proteins have yet been identified. U4 RNA sequence requirements for U4-U6 interaction and splicing have been analyzed in *Xenopus* oocytes (44). In the mammalian in vitro system, we have previously studied the importance of U4 RNA sequences in U4-U6 interaction and spliceosome assembly (45); however, this reconstitution system did not allow U4 RNA sequence requirements in splicing to be defined. The stem II region was found to be particularly important for U4-U6 interaction, whereas the 5′ stem-loop appears to have a U4-U6-destabilizing effect and is required for spliceosome assembly and splicing. A U4 mutant RNA with a large deletion of the entire single-stranded central region is functional in U4-U6 interaction but is splicing inactive (44). Binding of an antisense 2′-*O*-methyl (2′-OMe) RNA oligonucleotide in the same region, however, did not result in a defect of in vitro splicing (8). Binding of the common snRNP proteins (Sm proteins) involves the 3′-terminal stem-loop, the Sm-binding site (nucleotides 120 to 126), and the central stem-loop (27). In analogy to the U2 snRNP (30, 31), Sm protein binding of the U4 RNA is believed to be necessary for cytoplasmic cap trimethylation and nuclear snRNP transport; the m_3G cap and the Sm domain together are considered a bipartite nuclear localiza-

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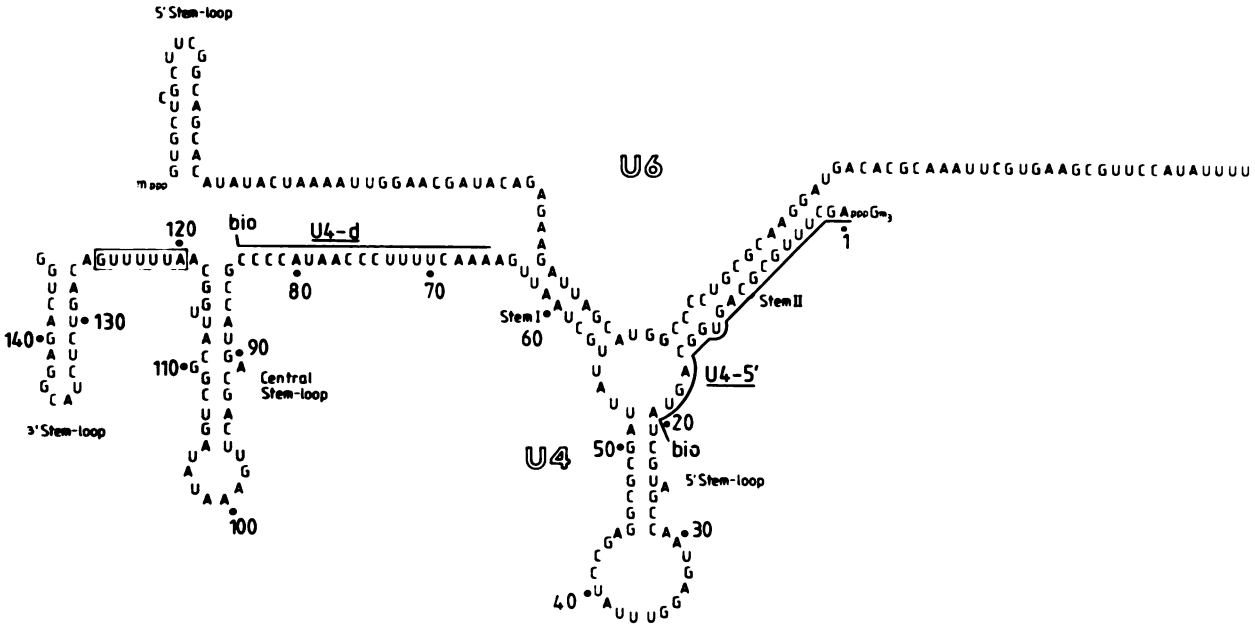


FIG. 1. Secondary structure model of the human U4/U6 RNA hybrid (3, 18, 23). The target sequences of the biotinylated antisense 2'-OMe RNA oligonucleotides (U4-5' and U4-d) and the Sm-binding site of U4 RNA (boxed sequence) are indicated.

tion signal of snRNPs (15, 16, 19). Mutations within the central and 3'-terminal stem-loops had no effect on U4-U6 interaction and splicing; when tested in vivo, a U4 RNA with an Sm-binding site deletion was nonfunctional in splicing, because nuclear transport was blocked (44). However, a large 3'-terminal deletion, removing the central stem-loop, the Sm-binding site, and the 3'-terminal stem-loop, efficiently assembled into spliceosomes in vitro (45). The question of whether Sm protein binding is essential for splicing therefore remained and can be addressed only in an in vitro system.

Here we report on the development of a splicing complementation system that allowed for the first time the biochemical analysis of U4 RNA functions in splicing in vitro; in addition, this system is useful for studying snRNP and spliceosome assembly steps preceding splicing and for investigating interactions of U4 RNA with other splicing factors. Following the specific depletion of U4 RNA from nuclear extract, splicing activity could be efficiently complemented by the addition of either affinity-purified U4 snRNP or synthetic U4 RNA. Using this complementation assay in combination with a large collection of new U4 mutant derivatives, we have mapped U4 sequences in the U4-U6 interaction domain and in the central region that are important in splicing and spliceosome assembly; surprisingly, binding of the Sm proteins was found to be not essential for splicing. In sum, our data demonstrate that several distinct U4 RNA regions contribute to the assembly of the U4/U6 snRNP, incorporation in a stable spliceosome, and splicing activity.

MATERIALS AND METHODS

DNA and 2'-OMe RNA oligonucleotides. DNA oligonucleotides were as follows: dT43, 5'-GCGAATTCGATTTAGG TGACACTATAG-3'; 491, 5'-GCGGCCAGTCTCCGTAGA GACTGTCAAAAATTGGGGTATTGGGAA-3'; 492, 5'-GC GGCCAATGCCGACTATA-3'; 2318, 5'-GCGGCCAGTCT CCGTAGAGACTGTCAAAAATTGCCAATGCCGACT ATATTTCAAGTCGTCATGGCGGGGTATTGGGAAAAT TAGCAATAATCG-3'; 2319, 5'-GCGGCCAGTCTCCGTA GAGACTGTCAAAAATTGCCAATGCCGACTATATTT CAAGTCGTCATGGCGGGGTATAAGTTTTCAATTAG C-3'; 2320, 5'-GCGGCCAGTCTCCGTAGAGACTGTCAA AAATTGCCAATGCCGACTATATTTCAAGTCGTCATG GCTGGGAAAAGTTTTCA-3'; 2843, 5'-GGTATTGGGAA AAGTAATTAGCAATAATCG-3'; 2844, 5'-GCGGGGTAT TGGGAATTTCAATTAGCAATA-3'; 2846, 5'-GCGGCCA GTCTCCGTAGAGACTGTCTACCATTGCCAATGCCG A-3'; and 2847, 5'-AGCAATAATCGCGCCTTACTCCAAA TAGGCTGGCTACGATACTGCC-3'. 2'-OMe RNA oligonucleotides (X denoting a biotinylated 2'-deoxycytidine [39]) were as follows: U4-5', 5'-UXXXXUACUGCCACUGCGC AAAGCU-3' (complementary to nucleotides 1 to 20 of human U4 RNA); and U4-d, 5'-UXXXXGGGGUAUUGGG AAAAGUUUC-3' (complementary to nucleotides 66 to 84 of human U4 RNA).

SP6-U4 mutant derivatives and transcription. SP6-U4 inv-5'loop corresponds to SP6-U4, with the region from nucleotides 29 to 44 inverted. It was derived from SP6-U4-EMBL8(+) [45] by site-specific mutagenesis, using oligonucleotide 2847 (24). SP6-U4 Δ64-67 and SP6-U4 Δ68-71 were constructed by site-specific mutagenesis, using oligonucleotides 2843 and 2844, respectively (24). SP6-U4 Δ64-71, SP6-U4 Δ72-77, and SP6-U4 Δ78-84 were constructed by the polymerase chain reaction (PCR) method, using the oligonucleotide pairs dT43/2318, dT43/2319, and dT43/2320, respectively, and SP6-U4 as a template. PCR-generated DNA fragments were cut with *Eco*RI and cloned in pEMBL8(+) between the *Eco*RI and *Hin*cII sites. SP6-U4 Δcentral stem-loop (nucleotides 85 to 118 deleted) and SP6-U4 3'Δ29 (nucleotides 118 to 145 deleted) were constructed by the PCR method, using SP6-U4 and the oligonucleotide pairs dT43/491 and dT43/492, respectively. SP6-U4 sub-Sm has nucleotides 122 to 125 (5'-UUUU-3') substituted by 5'-GGUA-3' and was derived from SP6-U4 by the PCR method, using SP6-U4 and the oligonucleotide pair dT43/

2846. The PCR-generated product was cut with *Eco*RI and ligated between the *Eco*RI and *Hinc*II sites of pEMBL8(+). SP6-U4 sub-Sm ΔstemII/2 was constructed by the PCR method, using SP6-U4 sub-Sm and oligonucleotides described earlier for SP6-U4 ΔstemII/2 (45).

The DNA templates for the synthesis of MINX pre-mRNA (49) and of SP6-U4, SP6-U4 ΔstemI, SP6-U4 ΔstemII, SP6-U4 ΔstemII/2, and SP6-U4 Δ5′stem-loop RNAs have been previously described (45). SP6-U4 RNA and all mutant derivatives carry two extra nucleotides at their 5′ ends (m^7GpppGA); SP6-U4, SP6-U4 ΔstemI, SP6-U4 ΔstemII, SP6-U4 ΔstemII/2, SP6-U4 Δ5′stem-loop, SP6-U4 inv-5′loop, SP6-U4 Δ64-67, and SP6-U4 Δ68-71 cut with *Dra*I yield RNAs with seven extra nucleotides at the 3′ end (AAUUUUU-3′); the other, PCR-generated transcriptional templates (SP6-U4 Δ64-71, SP6-U4 Δ72-77, SP6-U4 Δ78-84, SP6-U4 Δcentral stem-loop, SP6-U4 3′Δ29, SP6-U4 sub-Sm, and SP6-U4 sub-Sm ΔstemII/2) cut with *Hae*III give RNAs with no extra nucleotides at the 3′ end. A nonspecific RNA of 184 nucleotides containing polylinker sequences was synthesized by T7 transcription of *Nde*I-cut SP72. SP6 and T7 transcriptions were done in the presence of m^7GpppG cap analog and with [^{32}P]UTP as described previously (5).

U4 depletion of HeLa nuclear extract. HeLa cell nuclear extract (13) was centrifuged at 12,000 × *g* for 5 min at 4°C, and creatine phosphate and ATP were added to 5 and 0.5 mM, respectively. After the addition of biotinylated 2′-OMe RNA oligonucleotides U4-5′ and U4-d to final concentrations of 20 and 7 μg/ml, respectively, the extract was heated for 2 min to 41°C. Binding of 2′-OMe RNA oligonucleotides was allowed to proceed for 30 min at 37°C. The extract was then incubated again for 2 min at 41°C and cooled to room temperature. The KCl concentration was raised to 400 mM, and 0.5 volume of preblocked streptavidin-agarose beads was added (2), followed by gentle agitation at 4°C for 1 h. The streptavidin-agarose selection procedure was repeated once. Streptavidin-agarose was pelleted in a microfuge (3,000 rpm, 2 min at 4°C), and the depleted extract was dialyzed against 250 volumes of buffer D (13) for 3 h at 4°C. As a control, mock-depleted nuclear extract was prepared without adding oligonucleotides. The affinity purification of functional U4 snRNP has been described elsewhere (46).

Complementation of splicing activity. Affinity-purified U4 snRNP (approximately 4 ng of U4 RNA per μl) or U4 RNA derivatives were preincubated for 15 min at 30°C in a 1× splicing reaction mixture (25 μl) containing 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, 40 U of RNasin, 10 μl of U4-depleted or control extract, and 5 μl of dilution buffer (20 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.0], 100 mM KCl, 0.2 mM EDTA, 2 mM dithiothreitol); 4 ng of ^{32}P-labeled MINX pre-mRNA was added, and incubation was continued for 3 h. RNA was purified and analyzed on a denaturing 14% polyacrylamide–urea gel.

Spliceosome assembly. For spliceosome assembly, 25 ng of ^{32}P-labeled U4 RNA or mutant derivatives was preincubated in a 1× reaction mixture (25 μl) under splicing complementation conditions for 15 min. Then 100 ng of unlabeled MINX pre-mRNA was added, and incubation was continued for 20 or 40 min. In a control reaction (40 min), pre-mRNA was omitted. Heparin was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated for 5 min at room temperature. After a brief centrifugation (1 min at 12,000 × *g*), splicing complexes were analyzed by native gel electrophoresis (34). A marker for A and B complexes was obtained by incubation of 5 ng of ^{32}P-labeled MINX pre-mRNA in mock-depleted nuclear extract under splicing conditions.

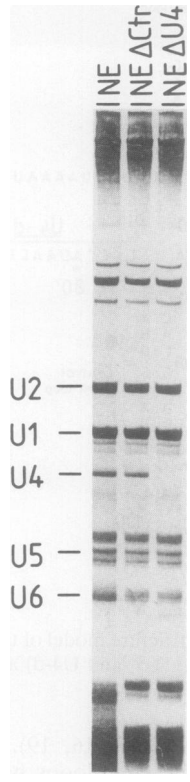


FIG. 2. U4-snRNP depletion of nuclear extract. Nuclear extract was depleted of the U4 snRNP as described in Materials and Methods. Total RNA was purified from equivalent aliquots (15 μl) of nuclear extract (NE), mock-depleted nuclear extract (control, NEΔCtr), and U4-snRNP-depleted nuclear extract (NEΔU4), followed by analysis through denaturing gel electrophoresis (8% polyacrylamide) and silver staining.

Sm protein binding and U4-U6 interaction. Immunoprecipitation assays with reconstituted snRNPs and the analysis of U4-U6 interaction were done as described previously (45).

RESULTS

Splicing complementation of a U4-depleted nuclear extract. To analyze the functions of U4 RNA in the mammalian splicing mechanism, we have developed an in vitro complementation system. Using a biotinylated 2′-OMe RNA oligonucleotide directed against stem II of U4 RNA (oligonucleotide U4-5′; Fig. 1), the U4-U6 interaction can be disrupted and U4 RNA/snRNP can be selectively removed from nuclear extract (8). To reproducibly achieve nearly quantitative depletion of U4, it was important first, to perform the binding reaction at a temperature at which the U4-U6 base pairing should be destabilized and second, to use, in addition to oligonucleotide U4-5′, a low concentration of U4d; the latter 2′-OMe RNA oligonucleotide is directed against the central domain of U4 (Fig. 1) and helped to deplete any residual U4. This procedure allowed the selective removal of U4 from nuclear extract, leaving U6 RNA/snRNP behind; the RNA analysis of the depleted extract and, most importantly, the reduction of its splicing activity to very low or undetectable levels demonstrated that the U4 depletion was

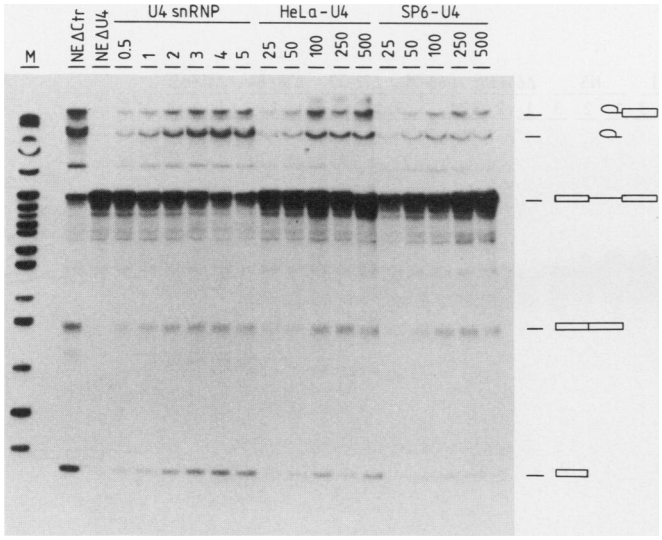


FIG. 3. Splicing complementation in U4-depleted nuclear extract with U4 snRNP, HeLa-U4 RNA, and SP6-U4 RNA. Affinity-purified U4 snRNP (titrated from 0.5 to 5 μl), gel-purified HeLa-U4 RNA, or synthetic SP6-U4 RNA (titrated from 25 to 500 ng) were incubated in U4-depleted nuclear extract, followed by the addition of ^{32}P-labeled pre-mRNA and a 3-h incubation under splicing conditions. In control reactions, the splicing activity in mock-depleted (NEΔCtr) and U4-depleted (NEΔU4) nuclear extract was assayed. ^{32}P-labeled pre-mRNA, splicing intermediates, and products (positions indicated on the right) were separated by denaturing gel electrophoresis. The band between the excised lariat and the pre-mRNA represents most likely a lariat degradation product. M, ^{32}P-labeled *Hpa*II-digested pBR322 fragments.

nearly quantitative (Fig. 2; compare lanes NEΔCtr and NEΔU4; for splicing activity, see below and Fig. 3).

We next tested whether splicing activity could be restored by complementation. U4-depleted nuclear extract was supplemented with U4 snRNP obtained in a functional form by affinity purification (46), with purified HeLa-U4 RNA, or with synthetic SP6-U4 RNA; complementation of splicing activity was measured as a function of the U4 snRNP/RNA concentration (Fig. 3). After U4 depletion of nuclear extract, splicing activity was consistently reduced to levels less than 5% of the control level (Fig. 3; compare lanes NEΔCtr and NEΔU4; see also Fig. 4A and B, lanes NEΔCtr and NEΔU4); compared with untreated nuclear extract, splicing activity of the control extract was not significantly reduced (data not shown). Addition of increasing quantities of the U4 snRNP (0.5 to 5 μl, corresponding to 2 to 20 ng of U4 RNA, per 25-μl reaction) to U4-depleted extract resulted in efficient restoration of splicing activity; saturation was reached at 3 μl of U4 snRNP, corresponding to 12 ng of U4 RNA, per 25-μl reaction (Fig. 3). Addition of increasing concentrations of either endogenous or synthetic U4 RNA (25 to 500 ng per 25-μl reaction) complemented splicing to similar maximal levels (Fig. 3), which were reached at a concentration of 250 ng of U4 RNA per 25-μl reaction. Splicing complementation activity was specific for U4 RNA, as the addition of nonspecific RNAs did not increase splicing activity above background levels (see below and Fig. 4). At the highest concentration of either the U4 snRNP or HeLa-U4 RNA, a small inhibitory effect on the second step of splicing became apparent (Fig. 3, lanes U4 snRNP/5 and HeLa-U4/500; compare levels of first exon and spliced product). Since we

TABLE 1. Splicing activities and assembly properties of mutant derivatives of human U4 RNA[a]

Mutant	Splicing	Spliceosome assembly	Sm
SP6-U4	++	++	++
SP6-U4 Δstem I	+	−	++
SP6-U4 Δstem II	−	−	++
SP6-U4 ΔstemII/2	−	−	++
SP6-U4 Δ5′stem-loop	+	−	++
SP6-U4 inv-5′loop	+	−	++
SP6-U4 Δ64-67	+	+	++
SP6-U4 Δ68-71	++	+	++
SP6-U4 Δ72-77	++	++	++
SP6-U4 Δ78-84	++	++	++
SP6-U4 Δcentral stem-loop	+	++	+
SP6-U4 3′Δ29	+	++	−
SP6-U4 sub-Sm	++	+	−
SP6-U4 sub-Sm ΔstemII/2	−	−	−

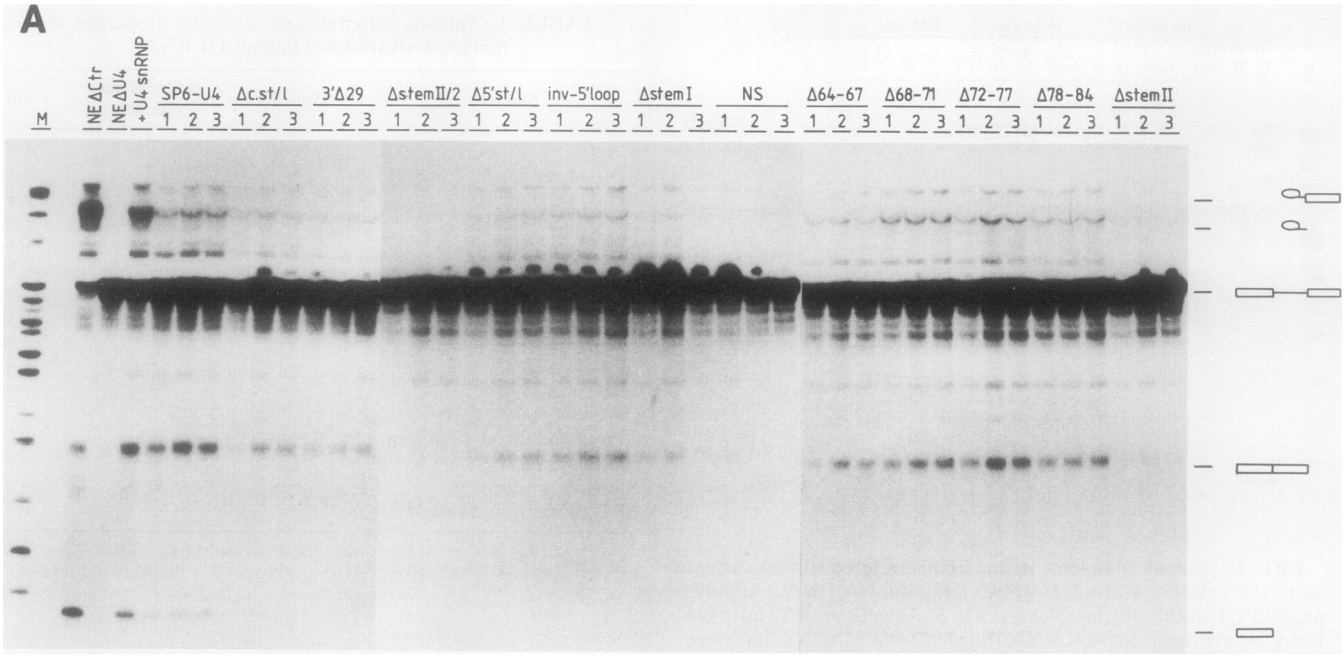
[a] Efficiencies are compared with those of wild-type SP6-U4 and are expressed as ++ (50 to 100%), + (10 to 50%), and − (background level).

estimate the U4 RNA concentration in the control splicing reaction to be approximately 1 μg/ml, splicing activity could be efficiently complemented with the U4 snRNP at ca. 50% of the U4 concentration present in a control splicing reaction. For comparison, a similar level of splicing complementation required the addition of U4 RNA at a concentration 10-fold above the endogenous level.

In sum, the U4 snRNP has a significantly higher splicing complementation activity than does U4 RNA. In part, this difference may be related to (i) the observation that only approximately 10% of U4 RNA assembles into Sm-immunoprecipitable snRNPs in the depleted extract and (ii) the different stabilities of the U4 snRNP and U4 RNA during the long incubation of the splicing complementation reaction (see below; data not shown). However, U4 RNA efficiently complemented splicing at increased concentrations, thus allowing the analysis of mutant derivatives.

The U4-U6 interaction domain: stem II of U4 RNA is splicing essential. First, we assayed the splicing activities of several U4 RNA mutant derivatives in which sequence elements of the U4-U6 interaction domain are deleted. Some of these mutant RNAs have previously been characterized for their ability to bind Sm proteins, to interact with U6 RNA, and to assemble into spliceosomes (45) (data are summarized in Table 1). The stabilities of these mutant RNAs in U4-depleted extract did not significantly differ from that of wild-type SP6-U4 RNA (data not shown). To detect even low splicing activities, each RNA was assayed after a 3-h incubation under splicing complementation conditions at three different U4 RNA concentrations (100, 200, and 300 ng per 25-μl reaction). The relatively long incubation period of 3 h resulted in partial degradation of the lariat intermediate and intron lariat RNAs (Fig. 4). As controls, splicing complementation was tested in mock-depleted nuclear extract (Fig. 4A and B, lanes NEΔCtr), and in U4-depleted nuclear extract alone, with U4 snRNP, wild-type SP6-U4 RNA, or a nonspecific RNA of 184 nucleotides (Fig. 4A and B, lanes NEΔU4, +U4 snRNP, SP6-U4, and NS, respectively).

Deleting either the entire stem II region of U4 RNA (nucleotides 1 to 16) or only the 5′ half of it (nucleotides 1 to 8) reduced splicing activities to background levels (Fig. 4A, lanes ΔstemII and ΔstemII/2). In contrast, a U4 mutant



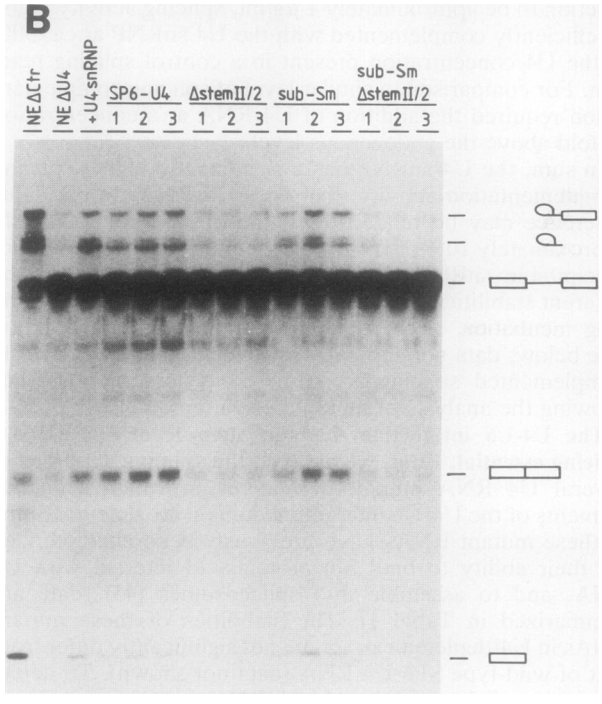


FIG. 4. Complementation of splicing activity by mutant derivatives of human U4 RNA. U4 RNA mutant derivatives (100, 200, and 300 ng of each, corresponding to lanes 1, 2, and 3, respectively) were assayed in U4-depleted nuclear extract for splicing complementation activity. In control reactions, the splicing activity in mock-depleted (NEΔCtr) and in U4-depleted (NEΔU4) nuclear extract as well as in U4-depleted nuclear extract complemented by a nonspecific RNA (NS, lanes 1 to 3) was assayed. For comparison, splicing complementation by U4 snRNP (3 μl per 25-μl reaction; lane +U4 snRNP) and by SP6-U4 RNA (SP6-U4, lanes 1 to 3) are also shown. ^{32}P-labeled pre-mRNA, splicing intermediates, and products (positions indicated on the right) were separated by denaturing gel electrophoresis (14% polyacrylamide). M, ^{32}P-labeled *Hpa*II-digested pBR322 fragments. Two different preparations of U4-depleted nuclear extract were used for the complementation reactions shown in panels A and B.

derivative lacking the stem I region (nucleotides 56 to 63) had a low residual splicing activity (Fig. 4A, lanes ΔstemI). Similarly, a deletion of the 5′ stem-loop (nucleotides 19 to 55) strongly reduced splicing activity (Fig. 4A, lanes Δ5′st/l). Substituting the loop of the 5′ stem-loop by inverting its sequence orientation strongly reduced splicing activity as well yet resulted in a higher residual splicing activity than did the mutant derivative with the entire 5′ stem-loop deleted (Fig. 4A, lanes inv-5′loop).

To correlate the splicing activities of these mutant RNAs with their spliceosome assembly properties, we also assayed them under splicing complementation conditions for the ability to mediate spliceosome assembly. Two complexes can be separated from each other by native gel electrophoresis: A, containing the U2 snRNP, and B, containing the U2, U4/U6, and U5 snRNPs (22). First, ^{32}P-labeled SP6-U4 RNA derivatives were added to U4-depleted nuclear extract and then unlabeled pre-mRNA to allow the assembly of splicing complexes, which were subsequently analyzed by native gel electrophoresis. For each U4 RNA derivative, spliceosome assembly was tested at two time points, 20 and 40 min, and, as a control, in the absence of pre-mRNA at 40 min (Fig. 5).

None of the three deletions of the stem I and stem II regions (SP6-U4 ΔstemI, SP6-U4 ΔstemII, and SP6-U4 ΔstemII/2) was detectably assembled into B complexes under splicing complementation conditions (data not shown), in accordance with previous data (45). When derivatives with either a deletion of the entire 5′ stem-loop (SP6-U4 Δ5′stem-loop) or a substitution of the 5′ loop (SP6-U4 inv-5′loop) were assayed, again no incorporation into complexes running at the characteristic position of the B complex was found; instead, heterogeneously migrating complexes of higher electrophoretic mobility were reproducibly observed and were not dependent on the presence of splicing substrate (Fig. 5, lanes Δ5′st/l and inv-5′loop). Since

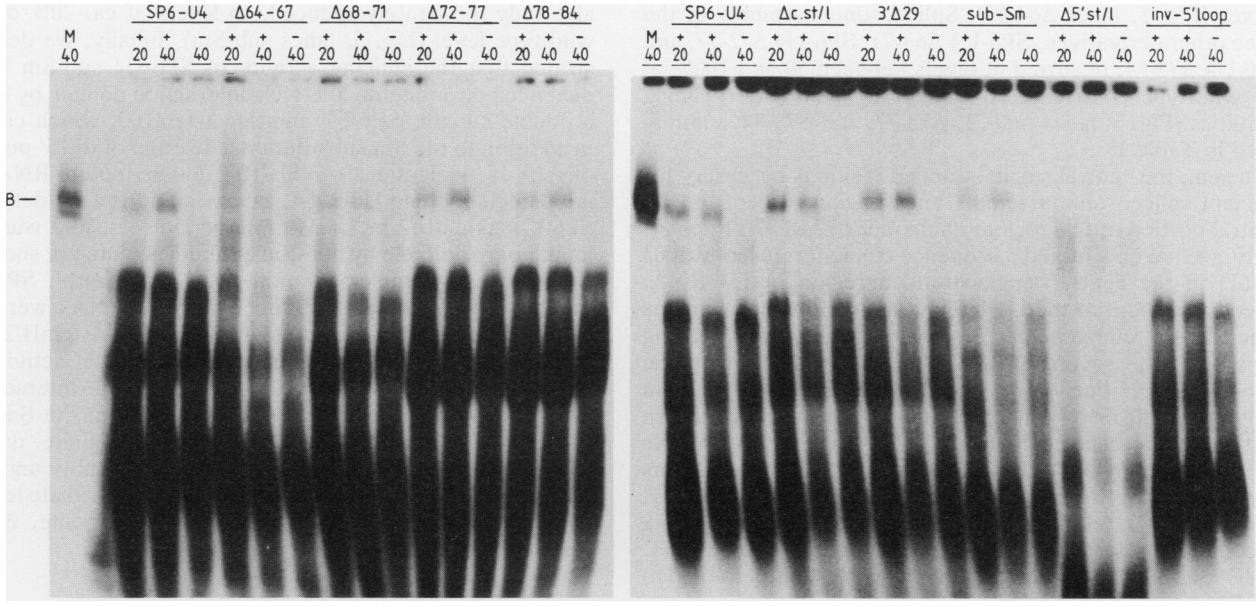


FIG. 5. Spliceosome assembly with mutant derivatives of human U4 RNA. U4 RNA mutant derivatives were preincubated in U4-depleted nuclear extract under splicing complementation conditions, followed by the addition of unlabeled pre-mRNA (lanes +) and incubation for the times indicated (20 and 40 min); as control, spliceosome assembly was assayed in the absence of added pre-mRNA (lanes −). To mark the position of the B complex, ^{32}P-labeled pre-mRNA was assembled into spliceosomes in mock-depleted nuclear extract (lanes M). The assembly of complexes was monitored by native gel electrophoresis.

these two mutant RNAs, however, supported splicing at significant levels (see above and Fig. 4A), they appear to assemble into spliceosomes that are not stable during native gel electrophoresis. Neither Sm protein binding, as measured by Sm immunoprecipitation (for SP6-U4 inv-5'loop, see Fig. 6, lanes inv-5'loop; for SP6-U4 Δ5'stem-loop, see reference 45), nor U4-U6 interaction was reduced for these two mutant RNAs (45; data not shown). In conclusion, our data demonstrate that within the U4-U6 interaction domain, the stem II region is essential for splicing; furthermore, the loop sequence of the 5' stem-loop is important for efficient splicing and for the assembly of a stable spliceosome.

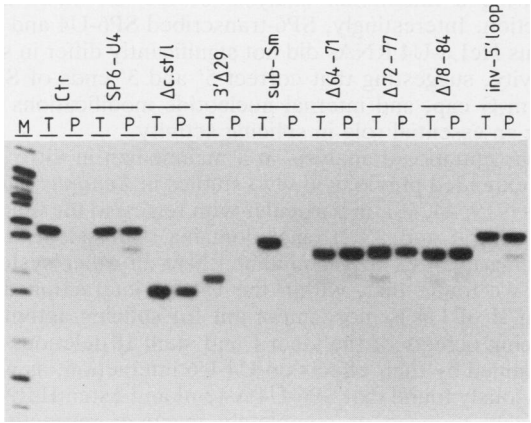


FIG. 6. Analysis of snRNP reconstitution by anti-Sm immunoprecipitation. ^{32}P-labeled SP6-U4 RNA and mutant derivatives were incubated under reconstitution conditions and immunoprecipitated with anti-Sm antibodies. For each RNA, 10% of the total reconstitution reaction (total, lanes T) and the anti-Sm-immunoprecipitated RNA (pellet, lanes P) are shown. In a control reaction, ^{32}P-labeled SP6-U4 RNA and nonimmune human serum were used (lanes Ctr). M, ^{32}P-labeled *Hpa*II-digested pBR322 fragments.

Mutational analysis of the central single-stranded region of U4 RNA. We next analyzed the single-stranded region within the central domain of U4 RNA (nucleotides 64 to 84) for spliceosome assembly and splicing functions. This region between stem I and central stem-loop was subdivided by five short deletions, each between four and eight nucleotides (Fig. 1). These mutant RNAs exhibited stabilities in U4-depleted extract similar to that of wild-type SP6-U4 RNA (data not shown). Splicing complementation assays indicated that deleting the region adjacent to stem I (SP6-U4 Δ64-71) severely reduced the splicing activity (data not shown). To delineate this important sequence element more precisely, two derivatives with smaller deletions, SP6-U4 Δ64-67 and SP6-U4 Δ68-71, were assayed for splicing complementation. The first of these, SP6-U4 Δ64-67, was affected most, reaching only approximately 20% of the splicing activity of wild-type SP6-U4; the second, SP6-U4 Δ68-71, was less strongly reduced, to a level of ca. 50% (Fig. 4A, lanes Δ64-67 and Δ68-71). The next deletion within the single-stranded region, SP6-U4 Δ72-77, resulted in very little reduction of splicing activity, reaching close to wild-type activity (Fig. 4A, lanes Δ72-77); finally, the 3'-most deletion of this series, SP6-U4 Δ78-84, was again more strongly affected in its splicing activity, being reduced to splicing levels of around 50% of the wild-type control level (Fig. 4A, lanes Δ78-84).

For each of these mutant derivatives, spliceosome assembly activities were also determined. A deletion of nucleotides 64 to 71 reduced spliceosome assembly most dramatically (data not shown), followed by the effect of deleting nucleotides 64 to 67; we noted in several experiments that SP6-U4 Δ64-67 RNA assembled at a very low efficiency with pre-mRNA into B complexes, which were obscured by heterogeneously migrating, pre-mRNA-independent com-

plexes (Fig. 5, lanes Δ64-67). Spliceosome assembly of the three other derivatives, SP6-U4 Δ68-71, SP6-U4 Δ72-77, and SP6-U4 Δ78-84, occurred at levels between 50 and 100% of the wild-type control level, correlating with their splicing activities (Fig. 5, lanes Δ68-71, Δ72-77, and Δ78-84; summarized in Table 1).

In sum, the central single-stranded region is necessary for efficient spliceosome assembly and splicing, except for the central portion of this region (nucleotides 72 to 77). Interestingly, we have identified a sequence element (nucleotides 64 to 67) in the central single-stranded region of U4 RNA, directly adjacent to the U4-U6 interaction domain, which is required for efficient spliceosome assembly and splicing activities. The severe effects of the short deletion in the SP6-U4 Δ64-67 derivative is not caused by defects in Sm protein binding or U4-U6 interaction; in fact, we have shown that the entire central single-stranded region is dispensable for Sm binding and U4-U6 interaction. Three deletions covering the entire single-stranded region (SP6-U4 Δ64-71, SP6-U4 Δ72-77, and SP6-U4 Δ78-84) did not alter Sm binding in comparison with wild-type SP6-U4 (Fig. 6, lanes Δ64-71, Δ72-77, and Δ78-84), nor did they impair the ability to bind to U6 RNA (data not shown).

The Sm domain of U4 RNA is not essential for splicing in vitro. The Sm domain of U4 RNA consists of the Sm-binding site (AU_5G, nucleotides 120 to 126) and the flanking central and 3′-terminal stem-loops (Fig. 1). To investigate a potential role of these sequence elements in splicing, we constructed several mutant derivatives: SP6-U4 3′Δ29, lacking the 3′ stem-loop and the Sm-binding site; SP6-U4 Δcentral stem-loop, in which the central stem-loop (nucleotides 85 to 118) is precisely deleted; and SP6-U4 sub-Sm, carrying a substitution (nucleotides 122 to 125; UUUU→GGUA) which should render the Sm-binding site nonfunctional. These mutant derivatives were less stable in U4-depleted extract than was wild-type SP6-U4; more than 20% of these RNAs remained after the 15-min preincubation period, compared with 40% of wild-type SP6-U4 (data not shown).

Figure 4 shows splicing complementation assays with these three U4 mutant RNAs. Deletion of either the 3′ stem-loop/Sm-binding site region (SP6-U4 3′Δ29) or of the central stem-loop (SP6-U4 Δcentral stem-loop) lowered the splicing activity to levels of around 20% of the wild-type level (Fig. 4A, lanes Δc.st/l and 3′Δ29). Despite their significant reduction in splicing activity, these two mutant derivatives assembled into spliceosomes at efficiencies comparable with those of wild-type SP6-U4 (Fig. 5; compare lanes Δc.st/l and 3′Δ29). Sm protein binding of the SP6-U4 Δcentral stem-loop RNA was only slightly reduced relative to wild-type SP6-U4 (Fig. 6; compare lanes SP6-U4 and Δc.st/l); as expected, deleting the 3′ stem-loop and the Sm-binding site (SP6-U4 3′Δ29) abolished Sm binding (Fig. 6, lanes 3′Δ29).

To determine the effect of Sm protein binding on splicing and spliceosome assembly, we used an Sm substitution mutant, SP6-U4 sub-Sm. As expected, Sm protein binding was undetectable (Fig. 6, lanes sub-Sm); in contrast, SP6-U4 sub-Sm RNA complemented splicing activity with a surprisingly high efficiency (ca. 50% of the wild-type level; Fig. 4B, lanes sub-Sm), suggesting that Sm protein binding, although necessary for optimal splicing complementation, is not essential for splicing activity. The same result was obtained with a U4 mutant derivative, SP6-U6 del-Sm, which carried a deletion of four uridines of the Sm-binding site and which did not detectably bind Sm proteins (data not shown). The spliceosome assembly activity of SP6-U4 sub-Sm RNA was also only moderately reduced, to levels of ca. 20% of the wild-type level (Fig. 5, lanes sub-Sm). Finally, we demonstrated that the splicing activity of SP6-U4 sub-Sm RNA depended on a functional U4-U6 interaction domain by using a double-mutant, SP6-U4 sub-Sm ΔstemII/2, which carries in addition to the Sm substitution a deletion of the 5′ portion of stem II (nucleotides 1 to 8). This double-mutant RNA did not detectably bind U6 RNA, as expected from the defect of SP6-U4 ΔstemII/2 in U4-U6 interaction (45), and was not immunoprecipitable by anti-Sm antibodies (data not shown). In splicing complementation activity, both SP6-U4 ΔstemII/2 and SP6-U4 sub-Sm ΔstemII/2 RNAs were reduced to background levels (Fig. 4B, lanes ΔstemII/2 and sub-Sm ΔstemII/2), confirming that the splicing activity of SP6-U4 sub-Sm does depend on an intact U4-U6 interaction. We conclude that structural alterations within the Sm domain, including inactivation of the Sm-binding site, do not have very strong effects on spliceosome assembly and stability and reduce splicing activity only to intermediate levels; the Sm domain, in particular the Sm-binding site, is not essential for splicing activity.

DISCUSSION

We have recently described a mammalian in vitro splicing complementation assay that allowed the functional analysis of U6 mutant RNAs in splicing and in all preceding assembly steps (46). Here we describe a similar complementation system for the functional analysis of U4 RNA, using U4-depleted nuclear extract. This system enabled us for the first time to systematically evaluate potential functions of U4 RNA in the mammalian splicing mechanism. When we compared the complementation activity of the U4 snRNP affinity purified from nuclear extract with that of U4 RNA, we found that for maximal splicing activity, approximately 20 times higher concentrations of U4 RNA than of the U4 snRNP were required. This difference is most likely due to the incomplete snRNP reconstitution of added U4 RNA in the extract and to a higher stability of the U4 snRNP during the long incubation of the splicing reaction. By the addition of relatively high concentrations of U4 RNA, however, efficient splicing complementation could be achieved, thus allowing a mutational analysis of the U4 RNA splicing function. Interestingly, SP6-transcribed SP6-U4 and endogenous HeLa-U4 RNAs did not significantly differ in splicing activity, suggesting that correct 5′ and 3′ ends of SP6-U4, the m_3G cap, and internal nucleotide modifications do not play an essential role in splicing activity.

Our mutational analysis in a mammalian in vitro system has extended previous in vivo studies in *Xenopus* and yeast cells (7, 9, 44, 48), in particular with regard to the function of the central and 3′-terminal domains. Consistent with the splicing analysis of U4 mutant RNAs in other systems (9, 44), we found that, within the U4-U6 interaction domain, stem II of U4 is most important for splicing activity. The splicing defects of the stem I and stem II deletions can be explained by their effects on U4-U6 interaction, as we have previously found that SP6-U4 ΔstemI and ΔstemII RNAs are strongly reduced or completely inactive, respectively, in their ability to assemble with U6 (45). Both a deletion of the entire 5′ stem-loop and a loop substitution did not completely abolish splicing in vitro, as a similar deletion did in vivo in yeast cells (9). This may reflect either different sensitivities of the in vitro and in vivo systems or differences between the yeast and mammalian splicing machineries. Two mutations in the 5′ stem-loop region led to the interest-

ing phenotype that they supported splicing at a low level yet did not assemble into detectable spliceosomes. A likely interpretation of this result is that these mutant spliceosomes are unstable during native gel electrophoresis. A sequence element important in spliceosome assembly and splicing was mapped in the central domain of U4, directly 3′ adjacent to the stem I (nucleotides 64 to 67). Significantly, this region is, besides the 5′ loop, most highly conserved (18) and, in the secondary structure model for free U4 RNA, lies within a loop (33). The spacing between the U4-U6 interaction and the Sm domains appears not to be critical, as at least one deletion derivative in this region, SP6-U4 Δ72-77, was similar to wild-type SP6-U4 in both spliceosome assembly and splicing activities.

With regard to the Sm domain (central stem-loop, Sm-binding site, 3′-terminal stem-loop), its structural integrity is not essential for splicing. On the basis of U2 snRNP protein binding and transport studies, it is established that the Sm domain plays a decisive role in the cytoplasmic trimethylation and cytoplasmic-nuclear migration of snRNPs (30, 31). Which of the protein components of the Sm domain contributes to each function is not known. Each of the spliceosomal snRNPs (U1, U2, U4/U6, and U5 snRNPs) carries its own set of Sm proteins; therefore, a considerable portion of the total mass of the spliceosome is made up of Sm domains. Whether the RNA or protein components of the Sm domain contribute, in addition, an essential splicing function has not been known so far. In yeast cells, a U2 mutant RNA with a large 3′-terminal deletion that includes the Sm-binding site could not be tested for functionality in vitro because of its instability (32). We have previously demonstrated that the entire Sm domain of U4 RNA is dispensable for spliceosome assembly in vitro (45); using the splicing complementation assay, we have clearly shown here that Sm protein binding is not essential for splicing activity in vitro. Splicing activity of the Sm mutant RNA, however, still depended on an intact U4-U6 interaction. The apparently lower splicing complementation activity of U4 mutant derivatives that are nonfunctional in Sm protein binding is most likely caused by their decreased stability. One should note that we have studied in vitro activities of mutant RNAs. In vivo, U4 RNA has to undergo, in addition to snRNP assembly, snRNA maturation and transport processes before it can function in splicing; this may explain some quantitative differences of mutant effects seen in vivo and in vitro. Our results suggest that the Sm proteins function primarily during snRNP maturation and transport, although in some cases they may play additional roles in providing protein-protein contacts (see, for example, reference 20). In this context it is also of interest that the snRNP-like spliced leader RNP of *Ascaris* sp., which is an essential *trans*-splicing component, requires an intact Sm-binding site for *trans* splicing (29). One or several of the *Ascaris* Sm proteins may have a specific *trans*-splicing function or may be required for the interaction of a component of the *trans*-splicing machinery with the spliced leader RNA.

In conclusion, our mutational analysis of U4 function demonstrated that U4 RNA is organized in multiple domains functioning during snRNP and spliceosome assembly. In addition to the role of the stem I and stem II regions in U4-U6 interaction and to the proposed function of the 5′ stem-loop during U4/U5/U6 multi-snRNP formation, we have identified the 5′ loop and part of the central single-stranded region as sequence elements important for splicing and spliceosome assembly. The Sm domain of U4 RNA is not required for splicing in vitro. In sum, the splicing machinery appears to be surprisingly flexible and tolerant to snRNA mutations; with the exception of stem II, no U4 RNA sequence was found to be essential for splicing in vitro, consistent with the hypothesis that U4 acts as a antisense negative regulator of U6 function (18).

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