Domain Analysis of Human U5 RNA

CAP TRIMETHYLATION, PROTEIN BINDING, AND SPLICEOSOME ASSEMBLY *

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We have analyzed the sequence requirements of the human U5 RNA during small nuclear ribonucleoprotein (snRNP) and spliceosome assembly. A collection of mutant derivatives of the human U5 RNA gene was constructed in a U1 expression vector and transiently transfected in mammalian cells. Using immunoprecipitation and affinity selection assays, the cap trimethylation, the binding of Sm proteins and of the U5 snRNP-specific protein p220, as well as the assembly of the U4/U5/U6 triple snRNP and of spliceosomes were determined. By mutational analysis we were able to assign distinct functions to several structural elements of the human U5 RNA. Efficient binding of the Sm proteins requires the 3′ stem-loop. Both the Sm protein-binding site and the 3′ stem-loop are necessary for the formation of the trimethyl guanosine cap, consistent with Sm protein binding being a prerequisite for cap trimethylation. Specific elements of the U5 RNA 5′ stem-loop contribute to efficient p220 association, in particular stem 1b. Interestingly, the highly conserved loop 1 appears to be a multifunctional element; in addition to its function in splice-site selection the 5′ loop is involved in binding of p220 and in the assembly of the U4/U5/U6 triple snRNP. In sum, this mutational analysis has identified four functional domains of the human U5 RNA.

Nuclear pre-mRNA splicing proceeds in a dynamic multicomponent RNA-protein complex, the spliceosome (for reviews, see Green, 1991; Krämer, 1995; Moore et al., 1993; Nilsen, 1994; Sharp, 1994). The assembly of the spliceosome and the two steps of the pre-mRNA splicing reaction require a large number of essential cofactors, among them four small nuclear ribonucleoproteins (snRNPs), 1 the U1, U2, U4/U6, and U5 snRNPs (for a review, see Lührmann et al., 1990). Among these the U5 RNA is of central importance, since, together with the U6 RNA, it is thought to be closely associated with the catalytic center of the spliceosome and may participate directly in splicing catalysis (for a review, see Weiner, 1993). Evidence for this has been derived from genetic, biochemical, and cross-linking studies in the yeast and mammalian systems (see below).

While the U5 RNA secondary structure is conserved, primary sequence conservation of the U5 RNA is surprisingly low. Extensive sequence comparisons revealed that besides the highly conserved loop 1 with nine invariant positions, only the internal loop 2 and the Sm protein-binding site near the 3′ end are conserved (Frank et al., 1994; Guthrie and Patterson, 1988). Deletion studies in yeast have defined a minimal functional U5 structure consisting of the 5′ loop and stem Ic, the internal loop 2 with an adjacent closing stem of any sequence, and the Sm domain (Frank et al., 1994; see Fig. 2).

In the course of the splicing cycle, the U5 RNA becomes part of several dynamic RNA-protein complexes, the U5 core complex, the 20 S U5 snRNP, and the 25 S U4/U5/U6 triple snRNP (Bach et al., 1989; Behrens and Lührmann, 1991); it is the triple snRNP form in which U5 is incorporated into the spliceosome.

Cross-linking and yeast genetic studies suggested that the U5 snRNP contributes to splice-site selection via both RNA-RNA and RNA-protein interactions. The U5 RNA interacts with the 5′ and 3′ splice sites; the U5-exon 1 interaction occurs already before the first step and helps to align the two exons during the second step of the reaction (Cortes et al., 1993; Newman and Norman, 1991; Newman and Norman, 1992; Sontheimer and Steitz, 1993; Wyatt et al., 1992). Studies of the U5 protein components have so far concentrated on the PRP8 protein from yeast and its mammalian homologue, p220. This largest known spliceosomal protein is splicing-essential (Jaksch et al., 1988) and highly conserved (Anderson et al., 1989; Hodges et al., 1995). Recent studies suggest that this protein may act in conjunction with the U5 RNA. It was detected in close contact with both splice sites and the branch point region and could be cross-linked to both pre-mRNA, lariat intermediate, and excised lariat, indicating that the U5 association with the splicing substrate is established early and persists throughout the splicing reaction (Garcia-Blanco et al., 1990; MacMillan et al., 1994; Teigelkamp et al., 1995a; Teigelkamp et al., 1995b; Whittaker and Beggs, 1991; Umen and Guthrie, 1995). An additional role of PRP8 in 3′ splice-site selection was suggested by Umen and Guthrie (1995).

Because of its dynamic interactions and the presence of so many protein components, the U5 snRNP may be the spliceosomal snRNP with the most complex structural organization. For binding of the Sm proteins two determinants were found to be important, the Sm binding site and internal loop 1 (IL1) (Jarmolowski and Mattaj, 1993). However, where specific proteins are bound, is less clear; there is only some evidence from protection experiments in mammalian extracts that the extended 5′ stem-loop structure of U5 may be involved (Bach and Lührmann, 1991; Black and Pinto, 1989). Finally, what interactions contribute to the association between the U4/U6 and the U5 snRNPs is completely unknown.

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1 The abbreviations used are: sn, small nuclear; RNP, ribonucleoprotein; 2′-Omé, 2′-O-methyl; PCR, polymerase chain reaction; IL1, internal loop; nts, nucleotides.
We have undertaken a mutational analysis of the human U5 RNA, using transient transfection of mammalian cells and several assays to determine cap trimethylation, Sm and p220 protein binding, as well as U4/U5/U6 triple snRNP and spliceosome assembly. As a result, we were able to map distinct functional domains of the U5 RNA.

**EXPERIMENTAL PROCEDURES**

DNA and 2'-O-Methyl (2'-OMe) RNA Oligonucleotides—DNA oligonucleotides are as follows. T7U5–5' AAGG-3'
TTTCTCTTCAG-3'; T7U5–3'; 5'-GGCGATCTAGTGTGCCAACGAGC-AAGCCCGTC-3'; U1ups-5'; 5'-GGCGACGTGGATTCACTGTTTGTTCCTCAAGACCCAGTTTGCGATCTAGTGTGCCAACGAGCTG-3'; SP3-primer, 5'-GGCGACGTGGATTCACTGTTTGTTCCTCAAGACCCAGTTTGCGATCTAGTGTGCCAACGAGCTG-3'; T7 promoter. A fragment was constructed from two oligonucleotides pUC-U1 (Ach and Weiner, 1987) (a kind gift from A. Weiner, Yale University, New Haven) as a template and oligonucleotides U1ups-5' and U2ds-3'.

The pGEM4 U1/U5 expression construct was generated by polymerase chain reaction (PCR) methods from three fragments. First, the upstream noncoding region of the U1 RNA gene was amplified, using pUC-U1 as a template and oligonucleotide pairs Sp6-primer/U2ups-3' and U2ds-5'/U2ds-3'. The amplified fragments were cut with EcoRI/Sall (upstream U1 fragment) and with Sall/HindIII (U5/U1 hybrid fragment) and ligated together into pGEM4. The pGEM4 U2/U5 expression construct was generated by PCR methods in two steps. First, a cassette vector including the U2 upstream noncoding region was cloned into the unique Sall restric- tion site of the U2 region by primer extension.

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The mutant U5 derivatives (see Table I) were generated through PCR methods, using pGEM4 U1/U5 as a template and mutagenic oligonucleotide pairs with the first four nucleotides of the U2 coding region and restriction sites for BamHI and HindIII.

**Analysis of Transient Expression—**

293 cells were grown on 96-mm dishes to 60% confluency, using 4% fetal bovine serum (Life Technologies, Inc.). Transfection was done at 48 h after transfection as described by Chirgwin et al. (1979). For the analysis of U5 RNA expression by primer extension (Cross et al., 1991), 5 µg of total RNA and 1-2 × 10⁶ cpm of oligonucleotides 25–6, 19–3, 47–28, or E4-CAT 5' end-labeled with [γ-32P]ATP were used per reaction.

For Northern blotting 5 µg of total RNA were separated on a 10% denaturing polyacrylamide-urea gel and probed with U5-specific primers 25–6, 19–3, and 47–28 (Wolff and Binder ef., 1992).

Immunoprecipitations—Nuclear and cytoplasmic extract were prepared from 293 cells 48 h after transfection as described by Lee et al. (1988). For anti-m5G immunoprecipitations 10 µl of nuclear and 15 µl of cytoplasmic extract were mixed, and RNA was isolated by proteinase K digestion and phenol/chloroform extraction. Immunoprecipitations were performed with a polyclonal rabbit anti-m5G antiseraqup supplied according to Bringmann et al. (1983); the expected specificity for m5G-capped snRNAs was demonstrated (data not shown). RNA was isolated from supernatant and pellet fractions and subjected to primer extension. Anti-Sm immunoprecipitations were carried out with 30 µl of nuclear extract according to Wolff and Binder ef. (1993). RNA isolated from 20% of the total reaction and from the pellet was analyzed by primer extension. For anti-p220 immunoprecipitations a rabbit anti- serum against a His-tagged fusion protein containing the C-terminal 50 kDa of human p220 was used (M. Moore, H. Luo, and G. Moreau, Brandeis University). 10 µl of antisemir was incubated with 25 µl of protein A-Sepharose beads (Pharma Biotech Inc.) in 700 µl of PBS buffer (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na2HPO4, 2.2 mM KH2PO4, pH 7.4) for 12 h at 4°C. The precoated beads were washed three times with 1 ml of IP100 buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM MgCl2, 0.5 mM diithiothreitol, 0.05% Nonidet P-40). 40 µl of nuclear extract were incubated for 2 h at 4°C with the beads resus- pended in 250 µl of IP100 buffer. The beads were washed three times with IP100 buffer, and immunoprecipitated RNAs were recovered by vortexing the beads for 5 min in 200 µl of SBD buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 300 mM NaAc, 10 mM EDTA, 1% SDS) and subsequent phenol/chloroform extraction. In addition, RNA from 8 µl of nuclear extract (representing 20% of the total reaction) was prepared. RNA isolated from 20% of the total and from the pellet was analyzed by primer extension.

Affinity Selection of Splicing Complexes—Biotinylated MINX pre-mRNA (Zillmann et al., 1988) was synthesized by in vitro transcrip- tion (Wolff and Binder ef., 1992), using 30% of bio-11-UTP (Enzo). To assay the assembly of U5 RNA derivatives into splicing complexes, 30% of MINX pre-mRNA was transcribed by RNA polymerase II from an extract prepared from transfected cells (Lee et al., 1988). Before transfection, 10 µl of antisemir was incubated with 150 ng of biotinylated 2'-OMe RNA oligonucleotide U6e (complementary to nucleotides 82–101 of hu- man U6 RNA) and 6 µl of MINX RNA (Wolff and Binder ef.). 10 µl of antisemir was incubated with 25 µl of protein A-Sepharose beads (Pharma Biotech Inc.) and washed two times at 4°C, each for 10 min with 500 µl of WB300 (20 mM HEPES, pH 8.0, 300 mM KCl, 0.5 mM diithiothreitol, 0.01% Nonidet P-40). The oligonucleotide-tagged snRNPs were bound to 25 µl of preblocked streptavidin-agarose during a 2-h incubation at 4°C. The streptavidin-agarose beads were pelleted and washed three times with 500 µl of WB300 each for 10 min at 4°C. MINX RNA was released by incubation in 200 µl of SBD buffer (100 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, 150 mM NaAc, 1% SDS, and 0.5 mg/ml proteinase K) for 45 min at 55°C and subsequent phenol/chloroform extraction. Released RNA was analyzed by primer extension.
RESULTS

Expression of Human U5 RNA and Mutant Derivatives in Mammalian Cells—To express the human U5 RNA in mammalian cells, we constructed expression cassettes on the basis of the human U1 or U2 snRNA genes and inserted the sequence of the human U5 RNA (see "Experimental Procedures"). A 10-nucleotide marker sequence was added at the 5′ end, which allowed us to distinguish the transfected U5 RNA from the endogenous U5 RNA by primer-extension analysis (for the structure of pGEM4 U1/5, see Fig. 1). The resulting plasmids, pGEM4 U1/5 and pGEM4 U2/5, were transiently transfected into 293 cells; expression was measured by primer extension, using U5 RNA-specific oligonucleotides. Since after transient transfection the U5 RNA was consistently expressed at least 10-fold higher from the pGEM4 U1/5 than from the pGEM4 U2/5 construct (data not shown), all subsequent mutant derivatives were made on the basis of pGEM4 U1/5. A series of mutant derivatives of pGEM4 U1/5 was generated, in which individual sequence or structural elements were deleted or substituted, with the aim not to disturb the overall secondary structure (Fig. 2 and Table I). Most of the mutations affect the extended 5′ stem-loop of U5 (Δ5′ loop, Δ20–57, Δ1IL, ΔL1L2, sub-Δ3IL1L2, sub-Δ3IL1; in addition, the 3′ stem-loop was deleted (Δ3′ stem-loop), and a four-nucleotide substitution was introduced into the Sm site (sub-Sm).

Initially the expression of these U5 mutant derivatives during transient transfection in 293 cells was determined (Fig. 3; summarized in Table II). Since a primer specific for the 3′ stem-loop proved very inefficient in primer extension, three different primers had to be used to detect the expression of the U5 RNA derivatives, depending on the mutation (see three panels in Fig. 3); the efficiency of detection by primer extension varied considerably between the three primers (compare lanes WT in the three panels of Fig. 3) but was taken into account by simultaneously detecting the endogenous U5 RNA as an internal control. To control for primer specificity, RNA was also analyzed from untransfected cells. To compare transfection efficiencies, cotransfections were done with pE4-CAT. We conclude that the expression of the mutant derivatives Δ5′ loop, sub-Δ3IL1, and sub-Δ3IL1L2 is similar to wild-type, that is, in the order of 25% relative to the endogenous U5 RNA expression. The expression of sub-3IL1L2 is reduced to ~50% the other mutant U5 RNAs (Δ20–57, Δ1IL2, sub-Δ5IL1L2, Δ1IL1, Δ3′stemV loop, and sub-Sm) are expressed at very low levels (less than 10% of wild-type).

To test whether the mutant U5 RNAs possess correct 3′ ends, Northern blot analysis was carried out (data not shown). Although the mutant U5 RNAs with very low expression could not be analyzed this way, the other U5 derivatives showed the expected length and should therefore carry correct 3′ ends. The very low expression levels of certain mutant RNAs may be due to a variety of factors, including RNA stability, cap methylation, protein binding, localization, or 3′ end formation. To avoid problems of low in vivo stability, an in vitro reconstitution system such as the one developed recently by Ségault et al. (1995) would provide a useful complementary approach. Although we were able to reconstitute U5 core snRNPs, no functional complementation of U5-depleted splicing extracts could be achieved under our conditions (data not shown).

U5 cap Methylation Requires the 3′-Terminal Domain—Previous studies have demonstrated for the U2 RNA that cap methylation depends on a functional Sm protein-binding site (Mattaj, 1986). Therefore, we tested whether U5 RNA mutations affected cap methylation. RNA was prepared from transfected cells and subjected to anti-trimethylguanosine (m3G) cap immunoprecipitation. To compare the trimethyl cap formation of the U5 derivatives, both the supernatant and the pellet fractions of each immunoprecipitation reaction were analyzed by primer extension; representative examples are shown in Fig. 4 (for a summary, see Table II). Between 50 and 80% of the endogenous U5 RNA were precipitated; the levels were similar for the transfected wild-type U5 RNA, as well as for the mutant derivatives Δ5′ loop, sub-Δ3IL1L2, Δ20–57, Δ1IL2, sub-Δ5IL2L2, sub-Δ3IL1L2, sub-Δ3IL1L1, and Δ1IL1 (Fig. 4 and data not shown). In contrast, the sub-Sm mutant RNA was not detectably immunoprecipitated (Fig. 4, lanes sub Sm), demonstrating that an intact Sm site is essential for U5 cap methylation. Finally, mutant Δ3′ stem-loop was trimethyl-capped with a reduced efficiency of ~50% (lanes Δ3′ stem/loop), indicating that the 3′ stem-loop of human U5 may be an additional sequence element required for efficient cap methylation.

In further experiments we found that Sm protein binding correlated with trimethyl capping. All the U5 mutant RNAs that were expressed at sufficient levels to be tested showed normal Sm protein binding (Δ5′ loop, Δ20–57, sub-Δ3IL1L2, Δ1IL2, sub-Δ5IL2L2, sub-Δ3IL1L2, sub-Δ3IL1L1; the levels of the mutant U5 RNAs ΔΔIL1 and sub-Sm were too low in this experiment); in contrast, deletion of the 3′ terminal stem-loop (Δ3′ stemloop) resulted in reduced Sm protein binding (data not shown; summarized in Table II).

In sum, an intact Sm protein-binding site is essential for cap methylation. An additional important sequence element is the 3′ stem-loop. No sequence requirements for cap methylation could be mapped within the extended 5′ stem-loop structure.

Binding of the U5 snRNA-specific Protein p220 Depends on Elements of the 5′ Stem-loop—The U5 snRNP contains at least seven specific protein components, among them the p220 protein, which is highly conserved and has been implicated in several essential functions in the splicing mechanism (for references, see Introduction). To obtain insight into functionally important domains in the U5 snRNP, we have examined the U5 mutant RNAs for their ability to associate with p220, using immunoprecipitation assays. Extracts were prepared from transfected cells; immunoprecipitations were done with an anti-p220 antiserum, and RNA was analyzed from the immunoprecipitates by primer extension (Fig. 5; for a summary of the PhosphorImager analysis, see Table II). The anti-p220 antiserum is a polyclonal rabbit serum specific for the C-terminal 50 kDa of human p220. Under these conditions the efficiency of immunoprecipitating the endogenous U5 RNA was between 30 and 40%. In comparison to the endogenous U5 RNA, the immunoprecipitation efficiency of transfected wild-type U5

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2. M. Moore, H. Luo, G. Moreau, unpublished data.
RNA was reduced to about half, possibly resulting from the additional marker sequence at the 5′ end of U5 (lanes WT). The strongest effect of the mutations came from deleting the internal loop 2 (DIL2) and substituting stem Ib (sub-stem Ib). In comparison to the transfected wild-type U5 RNA, the other mutations reduced p220 binding to intermediate levels (D59 loop, D20–57, sub-5′IL2, sub-Sm) or had no effect (sub-stem Ic, sub-3′IL1, D3'stem/loop); due to inefficient expression, the DIL1 mutant could not be analyzed.

This mutational analysis demonstrates that several discrete elements of the 5′ stem-loop contribute to efficient p220 association. Specifically, stem Ib is essential, and other elements contribute to efficient p220 binding.

Mutational Analysis of U4/U5/U6 Triple snRNP and Spliceosome Assembly—In order to analyze the assembly of U5 mutant RNAs into U4/U5/U6 triple snRNPs, a new assay based upon biotin-streptavidin selection was developed (see “Experimental Procedures”). U4/U5/U6 triple snRNPs were selected in extracts from transfected cells, using a biotinylated 2′-OMe RNA oligonucleotide specific for the 3′-terminal sequence of U6 RNA (U6-e) and streptavidin-agarose. Because of the instability of the triple-snRNP at high ionic strength (Cheng and Abelson, 1987; Lossky et al., 1987; Wolff and Bindereif, 1992) and because of nonspecific interactions at low ionic strength it was important to carry out the selections at an intermediate salt concentration (300 mM KCl). Under these conditions endogenous U4/U5/U6 triple snRNPs could be affinity-selected with an efficiency of between 10 and 15%; the selection was specific by the following criteria: first, it was dependent on the addition of biotinylated 2′-OMe RNA oligonucleotide; second, it was sensitive to heparin treatment (Wolff and Bindereif, 1992); third, if the extract was first fractionated on a glycerol gradient and the individual gradient fractions then used for affinity selections, U5 could be detected only in the 25 S region (where the U4/U5/U6 triple snRNP is concentrated) but not in the 20 S region (where U5 snRNPs fractionate) (data not shown).
The following characteristics of all U5 snRNA mutant derivatives are listed: expression level, m3G cap formation, Sm protein binding, p220 protein binding, U4/U5/U6 triple-snRNP, and spliceosome assembly. The expression levels are given relative to the wild-type construct pGEM4 U1/U5 (WT). The efficiency of m3G cap formation, Sm, and p220 protein binding as well as U4/U5/U6 triple-snRNP and spliceosome assembly of mutant derivatives are compared with those of endogenous U5 snRNA and indicated as + (60–100%), +/− (10–60%), +/− (<10%, but above background level) and − (background level). p220 protein binding, U4/U5/U6 triple-snRNP and spliceosome assembly were quantitated by PhosphorImager analysis. ND, not determined.

| U5 snRNA derivative | Expression | m3G cap | Sm* | p220 | U4/U5/U6 | Spliceosome |
|---------------------|------------|---------|-----|-------|---------|------------|
| WT                  | +          | +       | +   | + (57%) | + (95%) | + (94%) |
| Δ5' loop            | +          | +       | +   | + (21%) | + (52%) | + (47%) |
| Δ20-57              | +/−        | +/−     | +   | + (33%) | ND      | ND        |
| Sub-stem Ic         | +          | +       | +   | + (59%) | + (100%)| + (91%)   |
| ΔIL2                | +/−        | +/−     | +   | − (0%)  | ND      | ND        |
| Sub-5' IL2          | +/−        | +/−     | +   | + (31%) | ND      | ND        |
| Sub-stem Ia         | +          | +       | +   | − (0%)  | ND      | ND        |
| Sub-3' IL1          | +          | +       | +   | + (59%) | + (59%) | + (45%)   |
| ΔIL1                | +/−        | +/−     | +   | + (52%) | ND      | ND        |
| Δ3' stem/loop       | +/−        | +/−     | +   | + (52%) | ND      | ND        |
| Sub-Sm              | +/−        | −       | ND  | (27%)  | ND      | ND        |

*Data not shown.

Fig. 4. Analysis of m3G capping of U5 mutant derivatives by anti-m3G immunoprecipitation. RNA prepared from transfected cells was immunoprecipitated and analyzed by primer extension (S, supernatant fraction; P, pellet fraction). The positions of the primer (P) and the extension products from the endogenous (e-U5) and transfected (t-U5) U5 RNAs are indicated.

DISCUSSION

We have used transient expression assays and a mutational analysis of the human U5 RNA to study the domain structure of this spliceosomal RNA, which has been implicated in several functions of the splicing mechanism. Several structural elements of human U5 RNA were found to be necessary for efficient expression: both internal loops (IL1, IL2), the Sm protein-binding site, and the 3' stem-loop. Our mutational analysis demonstrated that these elements have important functions during U5 snRNP biogenesis and assembly into the spliceosome.

The three major conclusions of our study are summarized and discussed in the following. 1) Several structural elements of the extended 5' stem-loop of U5 RNA, in particular stem Ia, contribute to efficient p220 binding. 2) The 5' loop partici-
pates not only in splice site selection, as earlier studies have established, but is also necessary for efficient p220 binding and triple snRNP assembly. 3) Both the Sm site and the 3' stem-loop are important for Sm protein binding and cap trimethylation.

Earlier chemical modification and protection studies had shown that U5 snRNP proteins are bound within the extended 5' stem-loop of the U5 RNA (Bach and Lührmann, 1991; Black and Pinto, 1989). We have used anti-p220 immunoprecipitation assays to analyze a major U5 snRNP component, the p220 protein. We note that our approach cannot distinguish between a direct U5 RNA/p220 contact and an indirect, protein-mediated association. Interestingly, efficient p220 association with the U5 snRNP requires several elements of the 5' stem-loop.
for p220 binding points to a potential difference between the sequence requirements of the mammalian and yeast U5 snRNP assembly (compare with Frank et al., 1994). Surprisingly, the extensive deletion in the mutant RNA U5 Δ20–57 still allowed p220 association, although at a reduced level, whereas deleting only the internal loop 2 abolished p220 binding. Several explanations of this apparent discrepancy are possible, for example incorrect folding of mutant RNA ΔIL2.

Interestingly, substituting the 5' loop had a significant effect on p220 binding, suggesting a protein binding function of this highly conserved U5 element in addition to its role in splice-site selection. This result is consistent with an earlier study of Bach and Lührmann (1991) who demonstrated that in the 20 S U5 snRNP the 5' loop is less accessible to enzymatic digestion than in the U5 core particle. On the other hand, Black and Pinto (1989) have shown that the 5' loop is accessible to chemical modifications in the U4/U5/U6 triple snRNP. Therefore the 5' loop of U5 RNA may exist in different conformations, whether U5 occurs as free RNA, in the U5 snRNP, or in the U4/U5/U6 triple snRNP.

Both the internal loop 1, at least its 3' part, and the 5' loop are also necessary for efficient U4/U5/U6 triple snRNP and spliceosome assembly. This probably reflects the requirement of binding of the human p220 protein for triple snRNP and spliceosome assembly, as earlier demonstrated for the yeast homologue, PRP8 (1992). The effects of two mutations, Δ5' loop and sub-3' IL1, on splicing complex formation and U4/U5/U6 triple snRNP assembly correlate, suggesting that a correctly assembled U4/U5/U6 triple snRNP is a prerequisite for spliceosome formation.

Finally, our mutational analysis showed that the Sm binding site and the 3' stem-loop are important elements for cap trimethylation. As first demonstrated for the U2 RNA (Mattaj, 1986), binding of the Sm proteins is a prerequisite for cap trimethylation. Our data show that this conclusion holds also in the case of the human U5 RNA. A difference appears to exist between the mammalian and yeast U5 snRNP assembly in that the human U5 RNA requires, in addition to the Sm site, the 3' stem-loop for efficient Sm protein binding (this study), whereas in yeast the 3' stem-loop is dispensable (Frank et al., 1994).

In sum, this mutational analysis defined four functional domains of human U5 RNA. First, the 3' domain, including the Sm binding site and the 3' stem-loop, is necessary for Sm protein binding and cap trimethylation. Second, IL1 is involved in Sm protein binding (Jarmolowski and Mattaj, 1993) and U4/U5/U6 triple snRNP assembly (this study). Third, stem I is essential for the binding of specific proteins (p220) and appears to function in combination with other structural elements of the extended 5' stem-loop of U5 RNA. Finally, the 5' loop is a multifunctional element, involved not only in splice-site selection, but also in p220 binding.

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REFERENCES

Ach, R. A., and Weiner, A. M. (1987) Mol. Cell. Biol. 7, 2070–2079
Anderson, G. J., Bach, M., Lührmann, R., and Beggs, J. D. (1989) Nature 342, 819–822
Bach, M., and Lührmann, R. (1991) Biochim. Biophys. Acta 1088, 139–143
Bach, M., Winklemann, G., and Lührmann, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6038–6042
Barabin, S. M. L., Sproat, B. S., Ryder, U., Blencowe, B. J., and Lamond, A. I. (1989) EMBO J. 8, 4171–4178
Bassger, S. J., and Stietz, J. A. (1993) In The RNA World (Gesteland, R. F., and Atkins, J. F., eds) p. 359–381, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Behrens, S.-E., and Lührmann, R. (1991) Genes Dev. 5, 1439–1452
Black, D. L., and Pinto, A. L. (1989) Mol. Cell. Biol. 9, 3350–3359
Brüningmann, P., Rinke, J., Appel, B., Reuter, R., and Lührmann, R. (1983) EMBO J. 2, 1129–1135
Cheng, S.-C., and Abelson, J. (1987) Genes Dev. 1, 1014–1027
Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
Cortes, J. J., Sontheimer, E. J., Seiwert, S. D., and Stietz, J. A. (1993) EMBO J. 12, 5181–5189
Cross, M., Gündüz, A., Palfi, Z., and Bindereif, A. (1991) Mol. Cell. Biol. 11, 5516–5526
Frank, D. N., Röka, H., and Guthrie, C. (1994) Mol. Cell. Biol. 14, 2180–2190
García-BilAncio, M. A., Anderson, G. J., Beggs, J. D., and Sharp, P. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3082–3086
Green, M. R. (1991) Annu. Rev. Cell Biol. 7, 559–599
Guthrie, C., and Patterson, B. (1988) Annu. Rev. Genet. 22, 387–419
Hogades, P. E., Jackson, S. P., Brown, J. D., and Beggs, J. D. (1995) Yeast 11, 337–342
Jackson, S. P., Lossky, M., and Beggs, J. D. (1988) Mol. Cell. Biol. 8, 1067–1075
Jarmolowski, A., and Mattaj, I. W. (1993) EMBO J. 12, 223–232
Krämer, A. (1995) In PremRNA Processing (Lamond, A. I., ed) pp. 35–64, R. G. Landes Co., Austin, TX
Lee, K. A. W., Bindereif, A., and Green, M. R. (1988) Gene Anal. Tech. 5, 22–31
Lossky, M., Anderson, G. J., Jackson, S. P., and Beggs, J. (1987) Cell 51, 1019–1026
Lührmann, R., Kastner, B., and Bach, M. (1990) Biochim. Biophys. Acta 1087, 265–292
MacMillan, A. M., Query, C. C., Allerson, C. R., Chen, S., Verdone, G. L., and Sharp, P. A. (1994) Genes Dev. 8, 3008–3020
Mattaj, I. W. (1986) Cell 46, 905–911
Moore, M. J., Query, C. C., and Sharp, P. A. (1993) In The RNA World (Gesteland, R. F., and Atkins, J. F., eds) p. 303–357, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY
Newman, A., and Norman, C. (1991) Cell 65, 115–123
Newman, A. J., and Norman, C. (1992) Cell 68, 743–754
Nilson, T. W. (1994) Cell 78, 1–4
Ségault, V., Will, C. L., Sproat, B. S., and Lührmann, R. (1995) EMBO J. 14, 4010–4021
Sharp, P. A. (1994) Cell 77, 805–815
Sontheimer, E. J., and Stietz, J. A. (1993) Science 262, 1989–1996
Sproat, B. S., Lamond, A. I., Beijer, B., Neuner, P., and Ryder, U. (1988) Nucleic Acids Res. 17, 3373–3384
Teigelkamp, S., Newman, A. J., and Beggs, J. D. (1995a) EMBO J. 14, 2602–2612
Teigelkamp, S., Whittaker, E., and Beggs, J. D. (1995b) Nucleic Acids Res. 23, 320–326
Umen, J. G., and Guthrie, C. (1995) Genes Dev. 9, 855–868
Weiner, A. M. (1993) Cell 72, 161–164
Whittaker, E., and Beggs, J. D. (1993) Nucleic Acids Res. 21, 5483–5489
Woff, T., and Bindereif, A. (1992) EMBO J. 11, 345–359
Woff, T., and Bindereif, A. (1993) Genes Dev. 7, 1377–1389
Wu, J., and Manley, J. L. (1989) Genes Dev. 3, 1553–1561
Wyatt, J. R., Sontheimer, E. J., and Stitz, J. A. (1992) Genes Dev. 6, 2542–2553
Zillmann, M., Zapp, M. L., and Berget, S. M. (1988) Mol. Cell. Biol. 8, 814–821