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Evidence for a Prp24 binding site in U6 snRNA and in a putative intermediate in the annealing of U6 and U4 snRNAs

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A mutation (U4-G14C) that destabilizes the base-pairing interaction between U4 and U6 snRNAs causes the accumulation of a novel complex containing U4, U6 and Prp24, a protein with RNA binding motifs. An analysis of suppressors of this cold-sensitive mutant led to the hypothesis that this complex is normally a transient intermediate in the annealing of U4 and U6. It was proposed that Prp24 must be released to form a fully base-paired U4/U6 snRNP. By using a chemical probing method we have tested the prediction that nucleotides A40–C43 in U6 mediate the binding of Prp24. Consistent with the location of recessive suppressors in U6, we find that residues A40–C43 are protected from chemical modification in U4/U6 complexes from the U4-G14C mutant but not from the wild-type or suppressor strains carrying mutations in U6 or *PRP24*. Furthermore, we find that base-pairing is substantially disrupted in the mutant complexes. Notably, the base-paired structure is restored in recessive suppressors despite the presence of a mismatched base-pair at the U4-G14C site. Our results support the model that Prp24 binds to U6 to promote its association with U4, but must dissociate to allow complete annealing.

Key words: chemical structure-probing/Prp24/U4/U6 snRNP/U6 snRNA/yeast

Introduction

The excision of introns from nuclear precursor messenger RNA (pre-mRNA) requires the specific and ordered interaction of the pre-mRNA with five small nuclear RNAs (U1, U2, U4, U5 and U6), which are associated with proteins to form small ribonucleoprotein particles (snRNPs). U1, U2 and U5 form individual snRNPs (Guthrie and Patterson, 1988; Green, 1991; Rymond and Rosbash, 1992; Steitz, 1992; Moore *et al.*, 1993), whereas U4 is found base-paired to U6 in the U4/U6 snRNP (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985; Siliciano *et al.*, 1987; Brow and Guthrie, 1988). U6 is also found as a single particle (U6 snRNP; Figure 1A). In the U4/U6 snRNP (Figure 1B), phylogenetic and genetic evidence indicate that U4 and U6 interact via base-pairing, forming the two intermolecular helices stems I and II (Brow and Guthrie, 1988; Shannon and Guthrie, 1991). The existence of stem I in the U4/U6 particle is consistent with a previously identified psoralen crosslink (Rinke *et al.*, 1985). Data from mammalian *in vitro* and *in vivo* experiments (Blencowe *et al.*, 1989; Hamm and Mattaj, 1989; Bindereif *et al.*, 1990; Vankan *et al.*, 1990; Wolff and Bindereif, 1992, 1993), as well as yeast reconstitution data (Fabrizio *et al.*, 1989), support the idea that both stems are required for U4/U6 snRNP formation.

The U4/U6 snRNP appears to assemble onto the spliceosome concomitantly with U5, probably as a tri-snRNP. The formation of the tri-snRNP requires Prp8 (Lossky *et al.*, 1987; Brown and Beggs, 1992) and Prp4 (Banroques and Abelson, 1989). Prior to the first catalytic step of splicing, U4 is released or at least significantly destabilized from the spliceosome (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Yean and Lin, 1991). Based on genetic and biochemical evidence, U6 then pairs with U2 snRNA to form the bulged intermolecular helix I immediately adjacent to the branchpoint recognition region (Madhani and Guthrie, 1992). The stem II region of U6 is thought to isomerize into the intramolecular stem of U6 (Wolff and Bindereif, 1993; Fortner *et al.*, 1994). This structure is consistent with the identification of additional U6/U2 pairing between the U6 3′ end and U2 5′ terminal sequences (Hausner *et al.*, 1990; Datta and Weiner, 1991; Watkins and Agabian, 1991; Wu and Manley, 1991). Immediately 5′ of the U2/U6 helix I is the invariant ACAGA sequence, which base-pairs with the 5′ splice site (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993; Sontheimer and Steitz, 1993). Because these structures allow the juxtaposition of the 5′ splice site with the branchpoint nucleophile (see Figure 1C), they have been proposed to be elements of the catalytic core of the spliceosome (reviewed by Wise, 1993; Madhani and Guthrie, 1994; Nilsen, 1994; Sharp, 1994). After completion of catalysis, these helices presumably must be dissolved to allow reformation of stems I and II in U4/U6. Thus, the current picture of the spliceosome assembly cycle is one in which U4 and U6 snRNAs are structurally dynamic.

In view of the central role of U6 in the splicing reaction, it is essential to understand the structural transitions this snRNA undergoes. Of equal interest is the identification of *trans*-acting factors which mediate these conformational changes. One candidate for a factor involved in these transitions is Prp24. Prp24 is a putative RNA binding protein which contains three consensus RNA recognition motifs (RRM) and co-immunoprecipitates with U6 snRNA but not with U4 snRNA from wild-type cells (Shannon and Guthrie, 1991). Interestingly, in extracts from cells containing a mutation in U4 (G14C) that destabilizes stem II of the U4/U6 snRNP (Figure 1B), anti-Prp24 antibodies immunoprecipitate U4 with U6. These mutant cells exhibit a cold-sensitive (cs) growth phenotype. Cold-insensitive

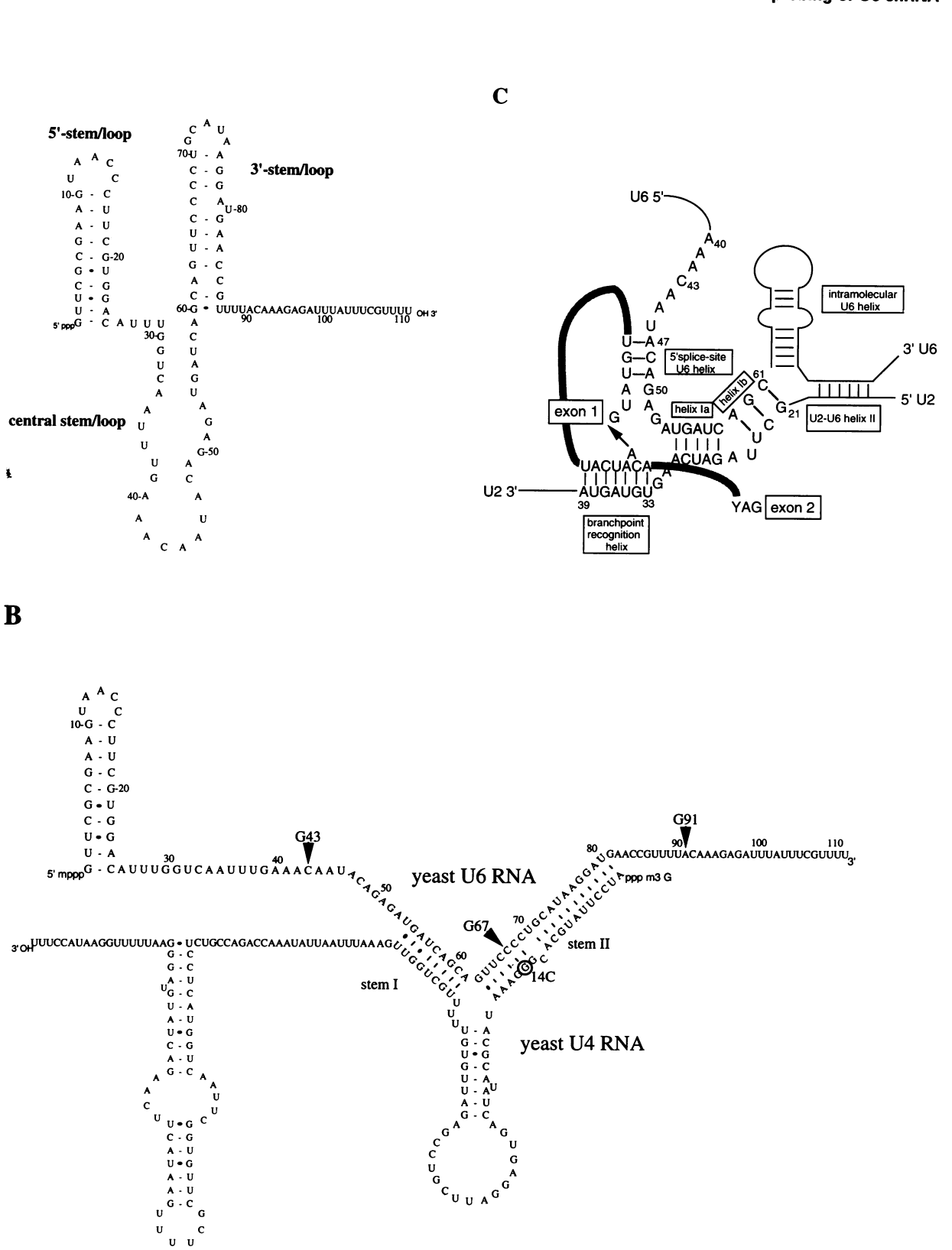

Fig. 1. Multiple conformations of *Saccharomyces cerevisiae* U6 snRNA. (**A**) Free U6 secondary structure, as proposed by Fortner *et al.* (1994). (**B**) Secondary structure of the U4/U6 complex (Brow and Guthrie, 1988). The circle indicates the position of the G14C mutation in the U4 snRNA and arrows show the position of the suppressor mutations in U6 (Shannon and Guthrie, 1991). (**C**) Shown are the five RNA−RNA helices in the spliceosome: (i) branchpoint recognition helix (Parker *et al.*, 1987), (ii) U2−U6 helix I (Madhani and Guthrie, 1992), (iii) U2−U6 helix II (Hausner *et al.*, 1990; Datta and Weiner, 1991; Watkins and Agabian, 1991; Wu and Manley, 1991), (iv) intramolecular helix in U6 (Wolff and Bindereif, 1993; Fortner *et al.*, 1994) and (v) the 5′ splice site−U6 helix (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993).

growth can be restored by suppressors which fall into three classes: (i) a dominant mutation in U6 that restores base-pairing in stem II (C67G), (ii) recessive mutations in two regions of U6 (U38C, A40G, C43G and A91G), and (iii) recessive mutations in *PRP24* (*prp24-2*, *prp24-3* and *prp24-4*; for location of the mutations in *PRP24* see Shannon and Guthrie, 1991). To explain these data it was proposed that the cs phenotype requires the destabilization of U4/U6 and results from the hyperstabilization of a competing complex containing U4, U6 and Prp24. The fact that sites of mutations in *PRP24* that suppress the cs phenotype correspond to amino acid residues known to be required for RNA binding in other RRM family members can most simply be explained by the model that Prp24 binds directly to the U6 snRNA. According to this hypothesis, the recessive suppressors in U6 would identify binding sites for Prp24. This conclusion is consistent with the observation that antibodies to Prp24 do not immunoprecipitate U6 or U4 in a cell extract made from the recessive suppressors tested (Shannon and Guthrie, 1991). To integrate these observations into the model of the splicing pathway, it was proposed that the U4/U6–Prp24 complex is normally a transient intermediate in the annealing of U4 and U6 snRNAs. The hyperstabilization of the U4/U6–Prp24 complex would inhibit the release of Prp24 and prevent the formation of a fully base-paired U4/U6 snRNP.

We set out to test a number of predictions arising from this hypothesis using biochemical methods. In particular, the model of Shannon and Guthrie (1991) predicts that the A40–C43 region of U6 is a protein binding site, and should thus be protected from chemical probing in free U6 complexes bound to Prp24, but not in wild-type U4/U6 complexes. Furthermore, the U4/U6-containing complexes in the U4-G14C mutant are predicted to be protected from modification in this region, due to the presence of Prp24, and this region should become accessible in cells with U6 and *PRP24* recessive suppressors. Finally, using chemical modification in combination with psoralen crosslinking, insights are revealed into the specific structure of the U4/U6–Prp24 complex and its potential role in assembly of the U4/U6 snRNP.

Results

Probing strategy

We have used a chemical footprinting technique to investigate the structure of U6 snRNA in free U6 and U4/U6 snRNPs from mutant and wild-type cells. This approach has been exploited very successfully in numerous RNA interaction studies (Inoue and Cech, 1985; Moazed *et al.*, 1986; Moazed and Noller, 1989). The major advantages of this approach are the single-nucleotide resolution and the fact that hundreds of nucleotides can be monitored in a single experiment. Two different chemical probes react with unpaired bases by modifying their Watson–Crick pairing positions: dimethyl sulfate (DMS) modifies N1 in A and N3 in C and kethoxal (KE) attacks N1 and N2 in G. This approach exploits the inability of reverse transcriptase to read through chemically modified bases, which results in a pause in the progression of the enzyme (Hagenbüchle *et al.*, 1978; Youvan and Hearst, 1979). Modified bases are identified by running four sequencing lanes on the same gel; unmodified control RNA, which has been subjected to identical treatment except for the omission of the chemical, is used to detect bands arising from strong secondary structures or nicks in the RNA template. We expected to be able to distinguish double-stranded regions from single-stranded ones in the probed RNA, based on the fact that base-paired nucleotides are not attacked by DMS or KE.

Comparison of the reactivity of bases in naked U6 snRNA and U6 snRNA from isolated wild-type U6 snRNPs and U4/U6 snRNPs

As demonstrated previously, three particles containing U6 RNA can be resolved by velocity sedimentation: U6 snRNP (Hamm and Mattaj, 1989; Bordonné *et al.*, 1990), U4/U6 snRNP and U4/U5/U6 snRNP (Black and Pinto, 1989; Bordonné *et al.*, 1990); in yeast, U6 is present in ~5-fold excess over U4 so that ~80% of it is not complexed with the U4 snRNA (Li and Brow, 1993). Predicting that the chemical footprint of the U6 snRNA would be different when U6 snRNA was probed in free U6 and U4/U6 snRNPs, we separated these particles via velocity sedimentation on a 10–30% glycerol gradient (Bordonné *et al.*, 1990). Wild-type splicing extract was incubated with ATP (see the legend to Figure 2) before fractionation and the sedimentation pattern was analyzed by Northern hybridization probing simultaneously for U4, U5 and U6 snRNAs. As shown in Figure 2A, the gradient allowed the separation of free U6 snRNPs (fractions 7–13), U4/U6 snRNPs (fractions 15–21) and U4/U5/U6 snRNPs (fraction 27).

We first took the peak fraction of the free U6 snRNP population and subjected it to chemical modification with KE and DMS (see Materials and methods). The modification data from probed 'naked' U6 snRNA (hot-phenol-extracted total yeast RNA) and free U6 snRNPs (Figure 3) are summarized in the secondary structure model of free yeast U6 snRNA proposed by Fortner *et al.* (1994) on the basis of *in vivo* modification experiments with DMS (Figure 4A and B). The model includes the terminal 5′ stem–loop, the phylogenetically non-conserved central stem–loop and the highly conserved 3′ stem–loop. When we compared the modification pattern of naked U6 snRNA (Figure 3, lanes 3 and 4) with the one derived from isolated free U6 snRNPs (lanes 2 and 5), we noticed that the 5′ stem–loop and the upper part of the 3′ stem–loop (C66–A79) appeared to be largely unaffected. Conversely, major changes were observed in the reactivity of particular nucleotides in the central domain of U6 (residues G30–G60) and the lower part of the 3′ stem–loop (G81–A83): nucleotides A40–C43, G50, G60, G81, A82 and A83 were reactive in naked U6 snRNA but appeared to be strongly protected in U6 snRNPs. Minor differences in the reactivity of the remaining nucleotides are most likely due to helix breathing. The proposed structural models for free U6 (Fortner *et al.*, 1994) and U4/U6 (Brow and Guthrie, 1988) would predict differences in the reactivity of bases between free U6 and U4/U6 snRNPs. Nucleotides in the central domain and the 3′ stem–loop in free U6 would have to be rearranged to form stem I and stem II in the U4/U6 particle. To test this, we isolated U4/U6 snRNPs from glycerol gradients and modified with KE and DMS (Figure 3, lanes 1 and 6). The data from the probed

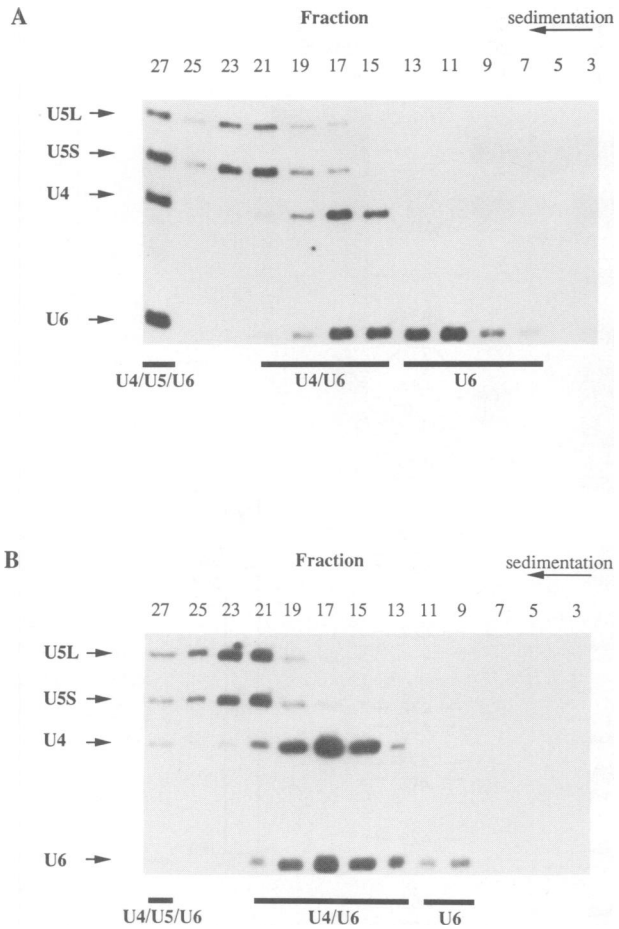

Fig. 2. Isolation of snRNPs via glycerol gradient sedimentation. Splicing extracts prepared from (**A**) the wild-type strain BJ2168 and (**B**) the U4-G14C mutant were incubated with 2 mM ATP, 2.5 mM $MgCl_2$ and 60 μM potassium phosphate (pH 7.0) for 20 min prior to fractionation on a 10–30% glycerol gradient. RNA was isolated from odd-numbered fractions and analyzed by Northern hybridization using probes specific for U4, U5 and U6 snRNAs. The direction of sedimentation, the fraction numbers and the positions of U6, U4/U6 and U4/U5/U6 snRNPs are indicated.

U4/U6 snRNPs are summarized in the proposed U4/U6 structural model of Brow and Guthrie (1988) in Figure 4C. We then compared the U4/U6 modification pattern (Figure 3, lanes 1 and 6) with that from free U6 snRNPs (Figure 3, lanes 2 and 5). Nucleotides G39, A40–C43, C48, A49, G50, A51, A53, G81 and A82–C85 were partially or completely protected in free U6, but were accessible to the chemicals in U4/U6 snRNPs. Conversely, nucleotides G30 and G31 were reactive in free U6 but were protected in U4/U6 snRNPs. All these nucleotides are located outside the proposed stem I and stem II regions of U4/U6. G55–A79 are nucleotides participating in the formation of stem I and stem II in the U4/U6 particle (Figure 4C, stems I and II). The significant but incomplete protection (Figure 3, lane 6) may be due to helix breathing. Moreover, we note the partial modification of nucleotides C68, C69 and G71. These stops occur in a region of stem II containing a mismatched base-pair; thus they may also be due to helix breathing. In yeast, it has been proposed that the U4/U5/U6 snRNP dissociates into U4/U6 upon incubation with ATP (Cheng and Abelson, 1987). We observed that the absence or presence of ATP caused no change in the individual modification patterns of U6 snRNA in free U6 and U4/U6 snRNPs under our conditions (data not shown).

Taken together, the data fit the prediction that nucleotides forming stem I and stem II in the U4/U6 snRNP would be protected from chemical attack as a result of base-pairing interactions. We are left to account for the difference between naked U6 snRNA, U6 snRNPs and U4/U6 snRNPs in nucleotides A40–C43. The observed protection in free U6 snRNPs could be due to RNA–RNA or RNA–protein interactions. To distinguish these alternatives, we incubated peak fractions from free U6 snRNP and U4/U6 snRNP populations with 1% SDS and 2 mg/ml proteinase K for 30 min at 37°C before chemical modification with DMS. Free U6 snRNPs that first showed a strong protection in this region (Figure 5A, lane 1) became accessible to the chemical after treatment with proteinase K/SDS (lane 3). Conversely, nucleotides A40–C43 from proteinase K/SDS-treated U4/U6 snRNPs remained reactive, and residues in stem I and stem II showed no change in reactivity (Figure 5B). In summary, the modification analysis suggests that the protection of A40–C43 in the free U6 snRNP may be due, directly or indirectly, to the interaction of a protein with the U6 snRNA and that this interaction seems to occur exclusively in the free U6 snRNP since the protection is not observed in the U4/U6 complex.

A cs U4 snRNA mutant contains an unusual U4/U6 snRNP complex

Shannon and Guthrie (1991) showed that U6 can be immunoprecipitated from isolated U6 snRNPs with anti-Prp24 antibodies in both wild-type and U4-G14C mutant extracts. In contrast, anti-Prp24 antiserum immunoprecipitated U4 and U6 from mutant but not from wild-type U4/U6 snRNPs. If the protection in the A40–C43 region is due to Prp24, the mutant free U6 snRNP modification pattern is expected to look like wild-type. In contrast, the pattern derived from the mutant U4/U6 snRNP would be different due to the presence of Prp24 in the mutant U4/U6 complex. Furthermore, the T_m of the mutant particle is ~37°C (Shannon and Guthrie, 1991) versus ~53°C measured in the wild-type strain (Brow and Guthrie, 1988). If these findings indicate that the U4/U6–Prp24 complex reflects an intermediate step in the assembly of U4/U6 snRNPs it might be expected to contain a partially annealed U4/U6 complex. Figure 2B shows the Northern blot of isolated mutant RNA from gradient fractions. The U6 snRNA from mutant free U6 and U4/U6 snRNPs was modified with KE and DMS. An autoradiograph of primer extensions is shown in Figure 6. We compared the modification patterns from wild-type and U4-G14C mutants with each other. The mutant free U6 snRNP (Figure 6, lanes 2 and 5) did not differ from wild-type U6 snRNPs (Figure 3, lanes 2 and 5). In contrast, the U4/U6 snRNP patterns looked very different from each other. As predicted, nucleotides A40–C43 were strongly protected in the mutant complex (Figure 6, lane 6), but were accessible in wild-type (Figure 3, lane 6). Conversely, G30 and G31 were reactive in the U4-G14C complex but were protected in the wild-type U4/U6 snRNP. Furthermore, nucleotides of stem II, which were protected in wild-type U4/U6

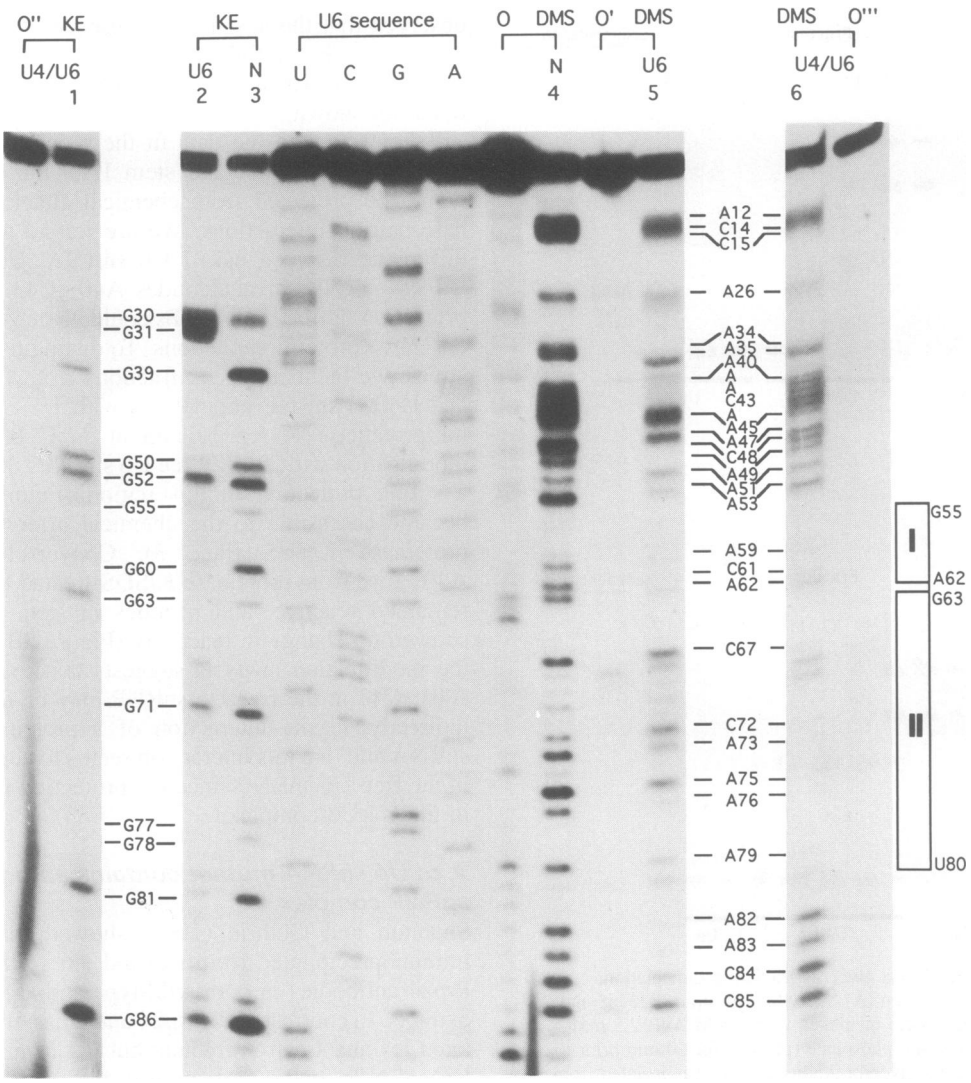

Fig. 3. Chemical modification of isolated wild-type snRNPs. Identification of chemically modified sites in U6 snRNA from isolated free U6 and U4/U6 snRNPs using the primer extension method with a U6-specific primer (see Materials and methods). Even-numbered glycerol gradient fractions corresponding to free U6 snRNPs and U4/U6 snRNPs were subjected to chemical modification with KE and DMS for 15 min at room temperature; N, naked U6 RNA (lanes 3 and 4); U6, free U6 snRNPs (lanes 2 and 5) and U4/U6, U4/U6 snRNPs (lanes 1 and 6). O, O′, O′′ and O′′′ are unmodified controls for naked U6 snRNA, free U6 and U4/U6 snRNPs, respectively. I and II, stem I and stem II in the U4/U6 snRNP. U, C, G and A are sequencing lanes and refer to the nucleotide sequence of the U6 snRNA. Modified positions are indicated by lines and numbered nucleotides.

snRNPs, were accessible in the mutant complex, especially nucleotides C67, G71, C72, A73, A75 and A79. This argues against the existence of an intact stem II in the mutant. The situation in stem I is less clear, since residues forming stem I in U4/U6 also participate in the formation of the upper part of the central stem in free U6. When we compared the modification patterns derived from the mutant free U6 snRNP (Figure 6, lanes 2 and 5) with the mutant U4/U6 snRNP (Figure 6, lanes 1 and 6), we observed that they were almost identical to one another, with the exception of nucleotides G39, A82 and A83 which were fully or partially protected in free U6 snRNPs but moderately modified in U4/U6 snRNPs. In addition, proteinase K/SDS treatment of both mutant free U6 and U4/U6 snRNPs rendered nucleotides A40–C43 susceptible to chemical attack (Figure 5C). This suggests that the protein that causes the protection in free U6 is also present in the mutant U4/U6 complex.

Suppression of the cs phenotype of the U4 snRNA mutant results in the restoration of a wild-type U4/U6 snRNP modification pattern

Based on immunoprecipitation experiments, Shannon and Guthrie (1991) predicted a model of suppression in which Prp24 has to be released from the U4/U6–Prp24 complex to allow reformation of the base-paired U4/U6 snRNP. If protection in A40–C43 of the U6 snRNA from wild-type free U6 and U4-G14C mutant U4/U6 snRNPs is due to the binding of Prp24, this region should become accessible in snRNPs from suppressor strains with mutations in U6 and *PRP24*.

Splicing extracts from these suppressor strains were fractionated on gradients and free U6 and U4/U6 snRNPs were subjected to chemical modification with DMS. The data are summarized in Tables I and II. Free U6 snRNPs (Table I) from wild-type (BJ2168), U4-G14C and the dominant suppressor U6-C67G exhibited a strong protec-

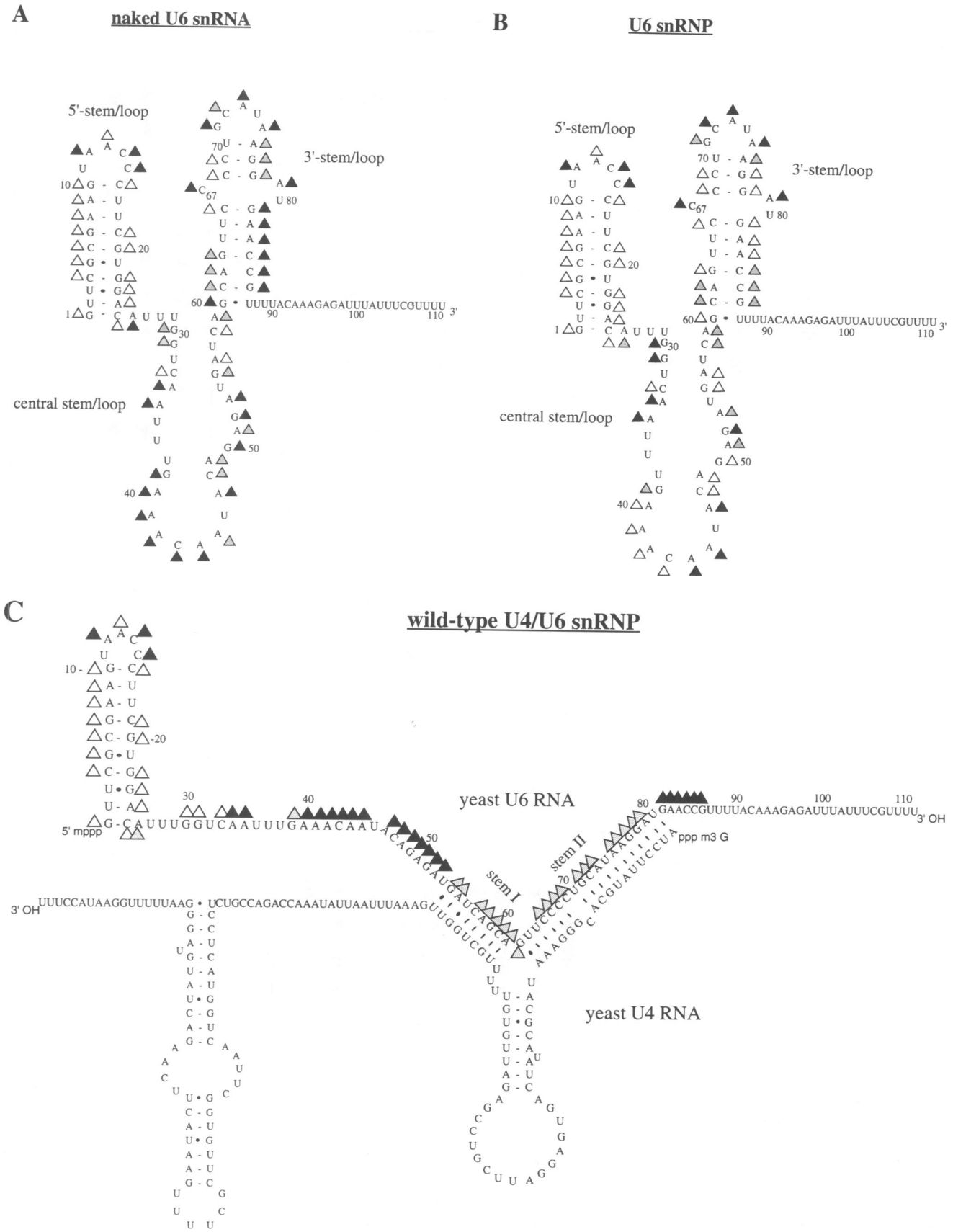

Fig. 4. Secondary structures of U6 snRNA. (**A**) Modification data from naked U6 snRNA are summarized in the proposed free U6 model (Fortner *et al.*, 1994). (**B**) Summary of DMS and KE modification data from free U6 snRNPs superimposed on the proposed model of Fortner *et al.* (1994). (**C**) Summary of DMS and KE modification data from U4/U6 snRNPs superimposed on the proposed model of Brow and Guthrie (1988). The relative reactivities represent the consensus of several independent experiments and are indicated as follows: open triangles, strongly protected; solid triangles, strongly reactive; shaded triangles, weakly reactive. U4, U6 snRNAs, stem I and stem II are indicated.

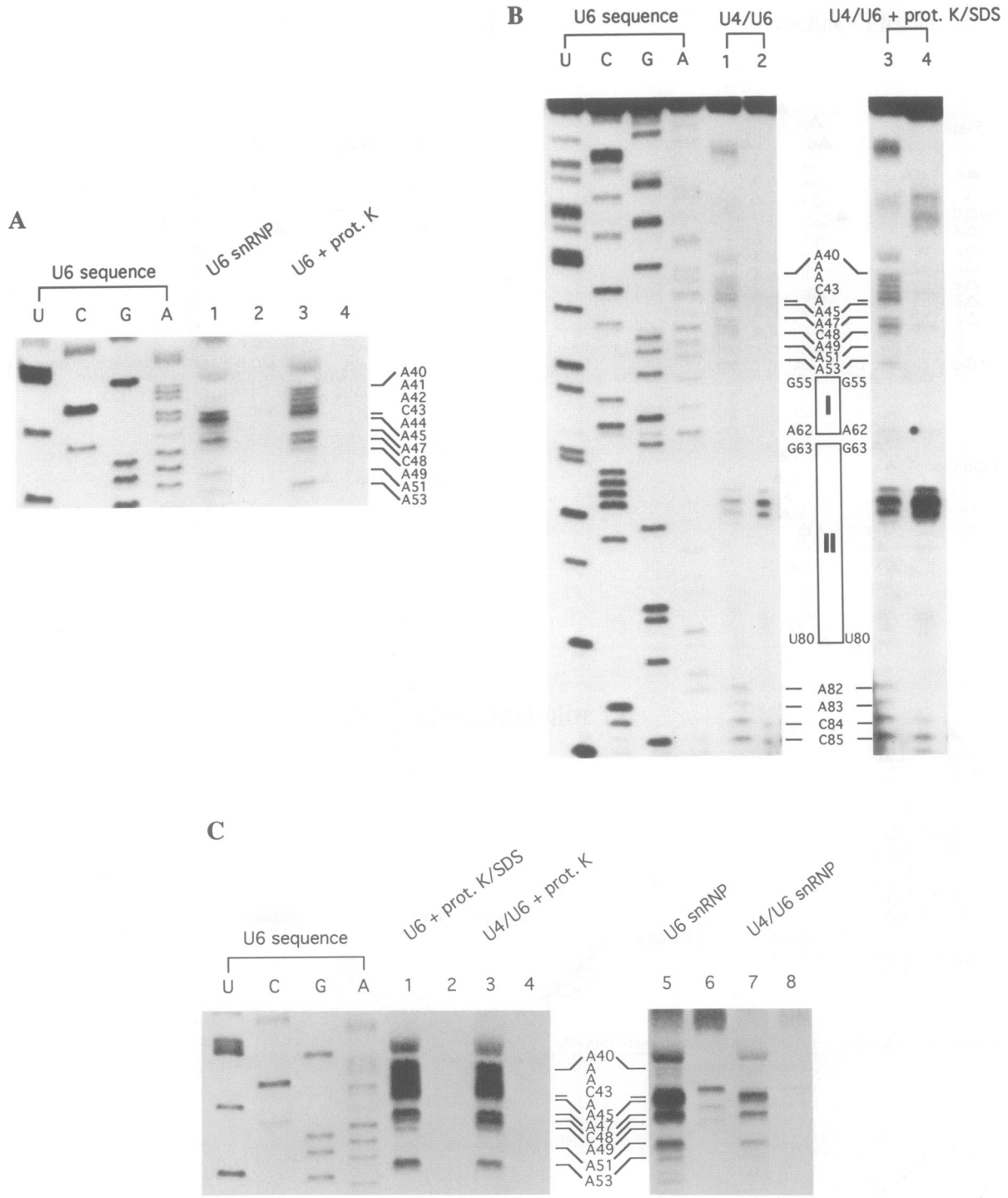

Fig. 5. Proteinase K/SDS treatment of U6 and U4/U6 snRNPs. Autoradiograph of DMS-modified wild-type free U6 snRNPs (**A**) and U4/U6 snRNPs (**B**) before (lane 1) and after (lane 3) proteinase K/SDS treatment (see text). (**C**) DMS-modified U4-G14C mutant free U6 and U4/U6 snRNPs after (lanes 1 and 3) and before (lanes 5 and 7) proteinase K treatment. I, II, stems I and II; lanes 2, 4, 6 and 8 are unmodified controls. (A and C) For clarity, only region C33–G55 of the U6 snRNA is shown.

tion in region A40–C43. In contrast, the recessive suppressor strains U6-C43G, U6-A91G, *prp24-2* and *prp24-4* lacked the protection of the same nucleotides. The results from probed U4/U6 snRNPs (Table II) showed suppressor strain modification patterns identical to wild-type: the abolished protection of A40–C43. Figure 7 shows an autoradiogram of primer extensions of modified U4/U6 snRNPs isolated from wild-type, U4-G14C, *prp24-2* and U6-C43G strains. Base-pairing in stem II seems to be significant but incomplete in these suppressors, as reflected by the partial protection of the participating nucleotides G63–A79; note that the probing experiments were carried out below the T_m of the U4 snRNA mutant (see Materials and methods). In summary, our findings strongly support the hypothesis that suppression is achieved by the destabilization of Prp24 from the U6 snRNA in region A40–C43

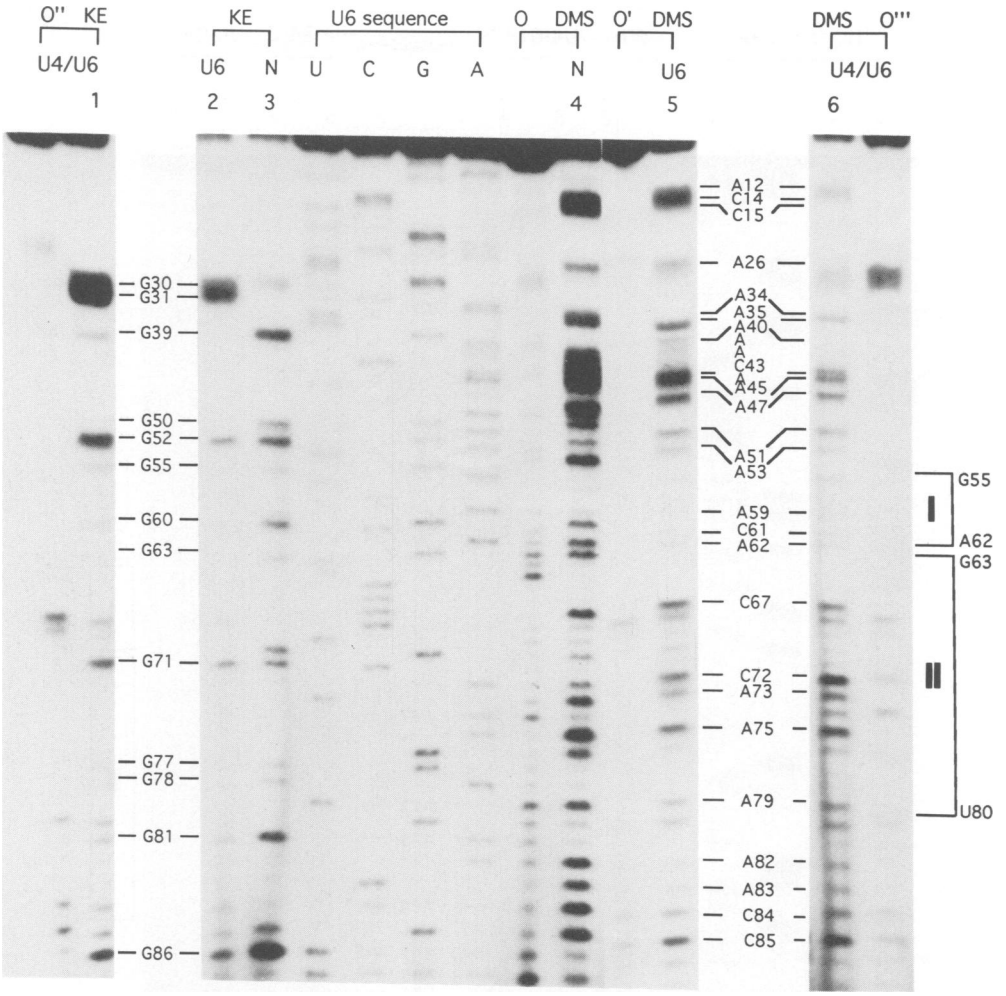

Fig. 6. Chemical modification of isolated U4-G14C mutant snRNPs. Autoradiograph shows KE- and DMS-modified sites in naked U6 snRNA (N, lanes 3 and 4), U6 snRNA from free U6 (U6, lanes 2 and 5) and U4/U6 snRNPs (U4/U6, lanes 1 and 6). O, O', O'' and O''' are unmodified controls. Other symbols are as described in the legend to Figure 3. All lanes are from the same experiment. Lanes O' and 5 are the result of a different exposure.

Table I. Summary of modification and immunoprecipitation data from free U6 snRNPs[a]

Strains	U4 allele	U6 allele	*PRP24* allele	Protection of nucleotides A40–C43	Co-immunoprecipitation of U6 snRNA with αPrp24
BJ2168	SNR14	SNR6	*PRP24*	+	+
U4-G14C	snr14-G14C	SNR6	*PRP24*	+	+
U6-C43G	snr14-G14C	snr6-C43G	*PRP24*	−	−
U6-C67G	snr14-G14C	snr6-C67G	*PRP24*	+	+
U6-A91G	snr14-G14C	snr6-A91G	*PRP24*	−	−
prp24-2	snr14-G14C	SNR6	prp24-2	−	−
prp24-4	snr14-G14C	SNR6	prp24-4	−	−

[a]Summarized are the DMS modification data from wild-type (BJ2168), the U4 snRNA mutant (U4-G14C) and suppressors of the U4-G14C mutation, containing mutations in U6 (U6-C43G, U6-C67G and U6-A91G), and *PRP24* (prp24-2 and prp24-4). +, protection from modification; −, loss of protection.

and the restoration of base-pairing in stem II in the U4/U6 complex.

Psoralen crosslinks U4 and U6 snRNA in wild-type and U4-G14C mutant strains

We have demonstrated a free U6 snRNP-like RNA secondary structure in mutant G14C-U4/U6 complexes (see above). The absence of stem II and the ambiguity of stem I in these complexes raised the question of how U4 and U6 are held together. We addressed this issue by irradiating nuclear extracts with 365 nm long-wave UV light in the presence of psoralen (see Materials and methods). Psoralen (4'-amino-methyl-4,5',8-trimethylpsoralen; AMT) is a chemical crosslinking reagent that intercalates within nucleic acid helices, and upon irradiation with long-wave UV light forms covalent bonds to pyrimidine residues juxtaposed on opposite strands. An intermolecular crosslink in the mutant complex would imply that U4 and

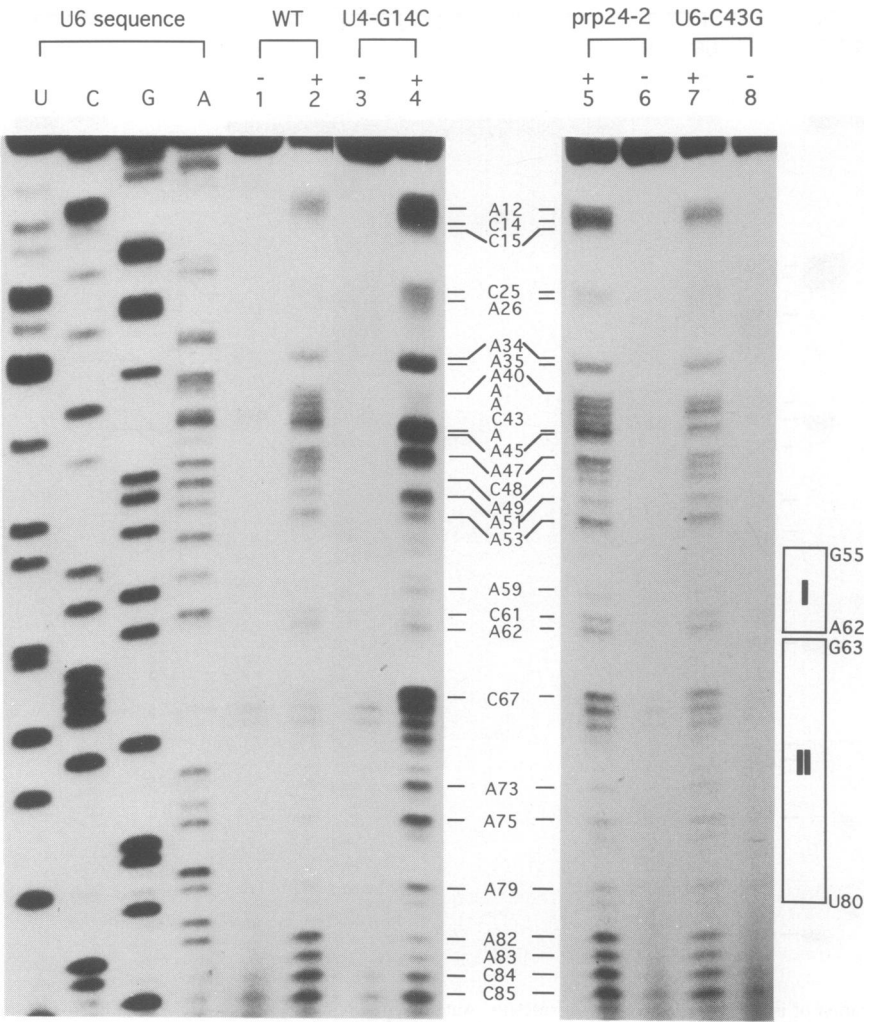

Fig. 7. DMS modification of U4/U6 complexes isolated from suppressor strains. Autoradiograph shows the sites of DMS modification (+) in U4/U6 snRNPs isolated from wild-type (WT), U4 snRNA mutant (U4-G14C) and the recessive suppressors in U6 (U6-C43G) and *PRP24* (*prp24-2*). Control reactions were carried out in the absence of DMS (−). Other symbols are explained in the legend to Figure 3. All lanes are from the same experiment. Lanes 3 and 4 are the result of a different exposure.

Table II. Summary of modification and immunoprecipitation data from U4/U6 snRNPs[a]

Strains	U4 allele	U6 allele	*PRP24* allele	Protection of nucleotides A40−C43	Co-immunoprecipitation with αPrp24 U4	U6	Formation of stem I	stem II
BJ2168	SNR14	SNR6	*PRP24*	−	−	−	+	+
U4-G14C	snr14-G14C	SNR6	*PRP24*	+	+	+	[b]	−
U6-C43G	snr14-G14C	snr6-C43G	*PRP24*	−	−	−	+	+
U6-C67G	snr14-G14C	snr6-C67G	*PRP24*	−	−	−	+	+
U6-A91G	snr14-G14C	snr6-A91G	*PRP24*	−	−	−	+	+
prp24-2	snr14-G14C	SNR6	prp24-2	−	−	−	+	+
prp24-4	snr14-G14C	SNR6	prp24-4	−	−	−	+	+

[a]See legend to Table I.
[b]The structure of the stem I nucleotides in U4-G14C is not absolutely clear.

U6 are in direct RNA−RNA contact. To test this, we performed experiments with splicing extracts prepared from wild-type and U4-G14C mutant strains. After crosslinking, the extracts were deproteinized and the psoralen-reacted snRNAs were analyzed by Northern hybridization. Crosslinking of U4 and U6 RNA should result in a new band migrating more slowly than U4 and U6 snRNA in the polyacrylamide gel. In fact, such bands were observed in both wild-type (Figure 8A, lane 2) and mutant extracts (lane 4) upon irradiation in the presence of psoralen. We identified the bands as the product of a crosslink between U4 and U6 snRNAs by sequentially probing a Northern blot for each snRNA (Figure 8A and B). The bands were not observed when psoralen was omitted (lanes 1 and 3). These results demonstrate that U4 and U6 snRNA interact directly in both wild-type and

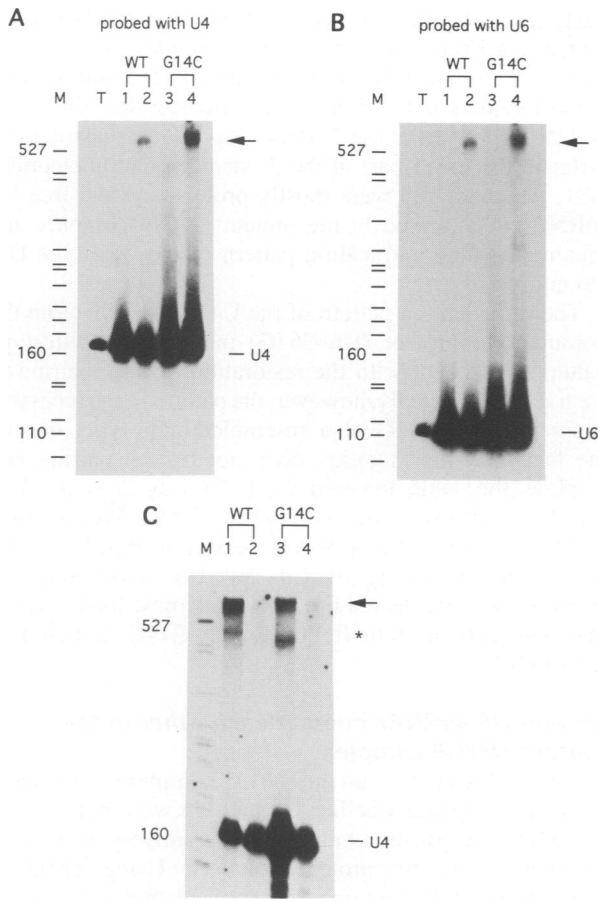

Fig. 8. Northern analysis of psoralen crosslinked snRNAs. (**A** and **B**) Irradiation of 30 μl nuclear extract prepared from wild-type and U4-G14C mutant strains in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of psoralen. Northern blots probed for U4 snRNA (A) or U6 snRNA (B). (**C**) Psoralen crosslinks in isolated U4/U6 (lanes 1 and 3) and U4/U5/U6 snRNPs (lanes 2 and 4) from wild-type and U4-G14C mutant strains. M, a labeled *Hpa*II digest of pBR325 was used to facilitate alignment. T, 10% of total splicing extract used in the experiment. RNAs were identified by Northern hybridization (see Materials and methods) and the positions of U4 and U6 snRNA are indicated. The arrow points to the position of crosslinked U4 and U6 snRNAs; bands denoted by the asterisk do not contain U6 snRNA and are thus considered to be a result of non-specific crosslinking of U4.

U4-G14C mutant strain. However, this U4−U6 crosslink could derive from U4/U5/U6 snRNPs in the total extract and not from the U4/U6 snRNP population. To determine whether the material we had chemically probed with DMS and KE produced this crosslink, we isolated snRNPs from glycerol gradients and crosslinked the entire U4/U6 snRNP and U4/U5/U6 snRNP populations separately. The blot was probed for U4 snRNA and the results are shown in Figure 8C. As can be seen, the majority of crosslinked U4 and U6 snRNAs was observed in the U4/U6 snRNP population (lanes 1 and 3). Less than 1% of total crosslink was contributed by the U4/U5/U6 population (lanes 2 and 4). Possible explanations of why we see less crosslink in the tri-snRNP are (i) regions on U4 and U6 snRNA that can be crosslinked in U4/U6 are not in close proximity to each other in the U4/U5/U6 snRNP, or (ii) proteins in the crosslinking region prevent the access of psoralen. In any case, from the above-mentioned experiments we conclude that the observed crosslink in our initial experiments with total splicing extracts represented crosslinks primarily in U4/U6 snRNPs.

Discussion

Distinct modification patterns of U6 snRNA from free U6 and U4/U6 snRNPs

Structural models for three different conformational states of U6 snRNA have been proposed previously: one for the free form of U6, one in which U4 and U6 are base-paired to each other via stem I and stem II, and one in which U6 is base-paired to U2 in the active site of the spliceosome (Figure 1; for references see Introduction). These structures presumably reflect sequential changes that occur during U4/U6 snRNP assembly and subsequent disassembly. A major challenge is to understand the relationship between these forms and to identify proteins that promote these transitions.

Using chemical modification we have investigated the molecular interactions in U6 snRNA in free U6 and U4/U6 snRNPs. The proposed models for the secondary structure of U6 in free U6 snRNPs (Fortner *et al.*, 1994; Figure 1A) and the U4/U6 complex (Brow and Guthrie, 1988; Figure 1B) argue that a large conformational switch in the 3′ stem−loop of U6 snRNA is required for assembly of the U4/U6 snRNP. Mutations that hyperstabilize the 3′ stem in the U6 snRNA have been shown to inhibit this conformational switch (Wolff and Bindereif, 1993; Fortner *et al.*, 1994). Our analysis of modification data from 'naked' U6 snRNA and free U6 snRNPs indicates that the secondary structure of the naked U6 snRNA closely approximates that found in the free U6 particle. The 5′ stem−loop and the upper part of the highly conserved 3′ stem−loop (Wolff and Bindereif, 1993; Fortner *et al.*, 1994) appear largely unaffected by the association of the U6 RNA with proteins during U6 snRNP assembly. In contrast, major changes in the reactivity of bases in the lower part of the 3′ stem and the phylogenetically non-conserved central stem−loop domain of the U6 RNA were observed, most evident in region A40−C43. After fractionation of snRNPs via glycerol gradient sedimentation, we found that the majority of free U6 snRNPs were strongly protected in this particular region against chemical attack. In contrast to our data, Fortner *et al.* (1994) reported that A40−C43 are not protected in U6 snRNA when living cells were probed with DMS, which they interpret to reflect the state of U6 snRNA in free U6 snRNPs. The simplest explanation for these differences is that *in vivo* the interaction of the protein with the U6 snRNA is not sufficiently stable to confer protection from DMS modification.

Our analysis of wild-type U4/U6 particles revealed the protection of nucleotides G55−A79. This observation is consistent with base-pairing in stems I and II in the proposed model of Brow and Guthrie (1988) and is supported by the fact that proteinase K/SDS treatment of U4/U6 snRNPs had no effect on the protection of these nucleotides. Notably, region A40−C43, which is strongly protected in free U6 snRNPs, is accessible to the chemical in the U4/U6 complex. Proteinase K/SDS treatment of free U6 snRNPs suggests that the protection of A40−C43 is due to the interaction of a protein with the U6 RNA either (i) directly through physical contact of A40−C43

with the protein or (ii) indirectly so that binding of the protein would alter the interaction of A40–C43 with some other region of the U6 snRNA. In any case, the best candidate protein would be Prp24, since it has been shown that it co-immunoprecipitates U6 snRNA in free U6 but not U4/U6 snRNPs from wild-type cells (Shannon and Guthrie, 1991).

U4/U6–Prp24, a putative annealing intermediate, exhibits properties distinct from U6 and U4/U6 snRNPs

Do U6 nucleotides A40–C43 mediate binding of Prp24? Shannon and Guthrie (1991) have shown previously that U4 becomes immunoprecipitable with anti-Prp24 antiserum in the U4-G14C mutant, while Prp24 could no longer co-immunoprecipitate with U6 or U4 in recessive suppressors of this mutation (U6-A91G, *prp24-2*). Only U6 from the dominant suppressor U6-C67G, a mutation that restores base-pairing in stem II, was co-immunoprecipitated. As predicted from these observations, we found that nucleotides A40–C43 were strongly protected in free U6 and U4/U6 snRNPs in the U4-G14C mutant strain (Figure 6, lanes 5 and 6), while these nucleotides were no longer protected against chemical attack in free U6 and U4/U6 snRNPs from the recessive suppressor strains. As expected, the dominant suppressor U6-C67G still exhibited protection in free U6 but not in U4/U6 snRNPs (Tables I and II). These results are particularly important because the U6 mutation C43G is located within the proposed binding site for Prp24 (Shannon and Guthrie, 1991). Interestingly, Fortner *et al.* (1994) isolated *cis*-acting suppressors (class II) of a 3′ stem hyperstabilization mutation that co-localize with the recessive U6 suppressors of Shannon and Guthrie (1991); one of them is C43U. Since suppressor mutations in *PRP24* reside within the conserved RNA binding motifs (RNP1 and RNP2) of Prp24, mutations in this domain are predicted to perturb the RNA binding ability of the protein. In summary, the specificity of suppression strongly suggests a direct interaction of Prp24 with the U6 snRNA in region A40–C43.

The structure of the U4/U6 complex in the U4-G14C mutant is distinct from wild-type U4/U6 snRNPs. Shannon and Guthrie (1991) have shown previously that the U4-G14C mutation, which disrupts base-pairing in stem II, lowers the T_m of the mutant G14C-U4/U6 complex from 53°C (wild-type) to 37°C. From this they concluded that the association of U4 with U6 must be at least partially disrupted in the mutant. Through chemical probing of U4/U6 snRNPs from the U4-G14C mutant we identified a structure of the U6 snRNA in these complexes that is dramatically distinct from that in wild-type U4/U6 snRNPs. Stem II appears to be destabilized in the mutant snRNP, which can be seen by the loss of protection of nucleotides G63–A79 (Figure 6, lanes 1, 2, 5 and 6). The interpretation of the data relating to stem I nucleotides (G55–A62) is ambiguous, since most of these residues were protected in both complexes (stem I in the U4/U6 snRNP and upper part of the central stem region in free U6 snRNPs). The only clear similarity between wild-type and mutant U4/U6 complex is found in region A82–G86: these residues were modified in both complexes. In contrast, the secondary structure of the U6 snRNA from mutant U4/U6 complexes closely resembles that obtained from free U6 snRNPs (Figure 6, lanes 1, 2, 5 and 6). This is most evident in the protection of nucleotides A40–C43 and the upper part of the 3′ stem–loop. The two structures differ in the lower part of the 3′ stem, in that nucleotides G81, A82 and A83 were mostly protected in the free U6 snRNP but modified in the mutant U4/U6 complex and thus match the modification pattern of the wild-type U4/U6 complex.

The modification pattern of the U4/U6 snRNP from the dominant suppressor (U6-C67G) matched the wild-type pattern, consistent with the restoration of base-pairing in stem II. Unexpectedly, however, the pattern in the recessive suppressor U6-A91G also resembled wild-type, despite the fact that this mutation does not restore pairing and displays the same lowered T_m (37°C) as does the U4-G14C mutant (Shannon and Guthrie, 1991). We conclude that the U6-A91G mutation, by weakening Prp24 binding, allows full annealing of U4 and U6, accounting for suppression. The decreased T_m is thus most likely due to the mismatch in stem II due to the G14C mutation in U4 snRNA.

U4 and U6 snRNAs contact each other in the mutant U4/U6 complex

Since the U4-G14C mutant U4/U6 complexes lack stem II, we investigated whether U4 and U6 were nonetheless in direct base-pairing contact or, alternatively, were held together solely by protein contacts. Using chemical crosslinking with psoralen, we showed that U4 and U6 snRNA in both wild-type and U4-G14C mutant extracts were directly juxtaposed. The band containing the crosslink in wild-type appears to differ slightly in mobility from the mutant (Figure 8A and B). As mentioned earlier, it has been shown previously in the mammalian system that U4 and U6 can be crosslinked in the upper part of stem I in the U4/U6 snRNP (Rinke *et al.*, 1985). We thus presume that the wild-type species corresponds to this contact. The location of the crosslink in the mutant complex is of obvious importance. However, because of technical difficulties we have not yet been able to map this site precisely.

Conclusions and perspectives

Although we cannot rule out the possibility that the G14C-U4/U6–Prp24 complex is a 'dead-end' product unique to the mutant, our data are fully consistent with the idea that it reflects a normally transient intermediate in the assembly of U4/U6 snRNP, as proposed by Shannon and Guthrie (1991). First, we have demonstrated that region A40–C43 of U6 is very likely to be a binding site for Prp24 in the U6 snRNP as well as in the novel U4/U6 complex which accumulates in the U4-G14C mutant. Moreover, our data indicated that U4 and U6 snRNA are at best only partially annealed in the G14C-U4/U6–Prp24 complex. Finally, we have shown that the recessive suppressors of the G14C mutation have restored base-pairing in stem II, despite the retention of a mismatched base-pair. This finding argues strongly that destabilization of Prp24 is necessary for full annealing of U4 with U6, as Shannon and Guthrie (1991) hypothesized.

An important unanswered question is the structural

basis of the apparently mutually exclusive relationship between the presence of Prp24 and the base-pairing status of stem II. One possibility is that the G14C mutation lies within a region of U4 that is part of a second RNA binding site for Prp24. This idea fits with the observation that Prp24 contains three RRM consensus motifs and is consistent with recent *in vitro* binding studies using purified Prp24, U4 and U6 snRNAs (A.Ghetti *et al.*, manuscript in preparation). According to this view, the normally transient interaction of Prp24 with this region would promote the formation of U4/U6 stem II in wild-type cells. The U4-G14C mutation could disrupt this second binding interaction, simultaneously hyperstabilizing an alternative structure of U4 which would make U4 unavailable as a pairing partner for U6. Prp24 would remain associated with this alternative structure in the U4/U6–Prp24 complex via binding to the U6 A40–C43 site and by the direct association of U4 snRNA with U6 snRNA indicated by our psoralen crosslinking data. An alternative explanation is that the U4-G14C mutation actually improves the affinity of the interaction with the second binding site of Prp24, perhaps indirectly stabilizing the interaction at the first site. We are currently attempting to determine the structure of U4 in the U4/U6–Prp24 complex to evaluate these hypotheses.

Materials and methods

Yeast strains

The haploid strain BJ2168 (MATa *leu2 trp1 ura3-52 prc1-1122 pep4-3 prc1-407 gal2*) is a protease-deficient strain (from the Yeast Genetics Stock Center, University of California, Berkeley, CA). The following strains were generated and described by Shannon and Guthrie (1991). The strain YKS2 (MATa *trp1 his3 ura3 ade2 lys2* snr14::TRP1 YCp50-SNR14) carries a disruption of the chromosomal copy SNR14, which is complemented by wild-type SNR14 on a YCp50 vector. snr14-G14C (MATa *trp1 his3 ura3 ade3 lys2* snr14::TRP1 pUN90-snr14-G14C) is a cs haploid U4 snRNA mutant strain derived from YKS2. snr6-A91G, snr6-C67G, snr6-C43G, *prp24-2* and *prp24-4* are various suppressor strains of snr14-G14C, containing chromosomal mutations in *SNR6* and *PRP24*, respectively.

Preparation of yeast nuclear extract, velocity sedimentation and Northern hybridization

All strains were grown to an OD_{600} = 0.8–1.5 in 2–4 l of YEPD medium. The extract was prepared according to Lin *et al.* (1985). Preparation and analysis of glycerol gradients were performed as described by Bordonné *et al.* (1990), except that 75 µl yeast extract were incubated with 2 mM ATP, 2.5 mM $MgCl_2$ and 60 µM potassium phosphate (pH 7.0) for 20 min at room temperature. The incubation mixture was then diluted 1:2 with HEPES buffer (80 mM HEPES/KOH, pH 7.9, 5 mM $MgCl_2$, 25 mM NaCl) and layered onto a 10–30% (w/v) glycerol gradient. RNAs were recovered from the fractions with phenol-chloroform and Northern blot analysis was performed according to Bordonné *et al.* (1990). Oligonucleotides used as probes: $14B_{137-158}$ (5′-AGGTATTCCAAAAATTCCCTAC) complementary to U4 snRNA, $6D_{112-92}$ (5′-AAAACGAAATAAATCTCTTTG) complementary to U6 snRNA and $7wtSmNR_{158-180}$ (5′-AAGTTCCAAAAAATATGGCAA-GC) complementary to U5 snRNA (Patterson and Guthrie, 1987).

Primer extensions and RNA sequencing

Total yeast RNA was prepared using the guanidinium thiocyanate method (Wise *et al.*, 1983). Primer extensions were performed by the method of Frank and Guthrie (1992). RNA sequencing was performed as follows: 12 µg of total cellular RNA and 2 ng of ^{32}P 5′ end-labeled oligonucleotide ($5\times10^5-10^6$ c.p.m.) primer were mixed and dried down under vacuum. The mixture was redissolved in 12 µl 1× annealing buffer (50 mM Tris–HCl, pH 8.3, 60 mM NaCl, 10 mM DTT), incubated for 3 min at 68°C, quick chilled on dry ice and allowed to thaw at room temperature. For each primer–template mixture, four tubes (A, G, C and T) were prepared. Each tube contained 1 µl 5× ddNTP mix (2 mM ddNTP in 1× annealing buffer) and 2 µl 5× dNTP mix (a mixture of all four dNTPs at 2 mM each in 1× annealing buffer). One µl of a reverse transcriptase mix containing 1 µl Life Science AMV reverse transcriptase (17 U/µl), 1.25 µl 5× reverse transcriptase buffer [250 mM Tris–HCl, pH 8.3, 300 mM NaCl, 50 mM DTT, 150 mM $Mg(OAc)_2$] and 4 µl diethylpyrocarbonate-treated H_2O were added to each NTP tube and the reactions were incubated at 37°C for 5 min before transferring to 42°C. The reaction was stopped by adding 5 µl stop-loading buffer and placing the tubes on ice. Prior to loading on a 6–8% polyacrylamide gel, the samples were denatured at 90°C for 3 min.

In vitro DMS and KE modification

This procedure has been described previously (Moazed *et al.*, 1986) for the modification of 16S rRNA, and has been modified for structure probing of snRNAs as follows: 1 µl concentrated DMS (Aldrich) and 40 µg *Escherichia coli* tRNA were added to one fraction (250 µl) from a glycerol gradient in HEPES buffer and incubated at 23°C with gentle agitation for 15 min. The reaction was stopped by the addition of 50 µl DMS stop solution (1 M Tris–acetate, pH 7.5, 1 M β-mercaptoethanol, 1.5 M NaOAc, 0.1 mM EDTA) and 2.5 vol of 96% EtOH, and was precipitated at −70°C for at least 10 min. The RNA was recovered by centrifugation, washed with 70% EtOH and vacuum-dried. Pellets were resuspended in 300 µl extraction buffer (0.5% SDS, 0.3 M NaOAc, 0.1 mM EDTA) and extracted twice with phenol–chloroform (50:50) and once with chloroform. After EtOH precipitation, the RNA was redissolved in 5 µl diethylpyrocarbonate-treated H_2O.

KE modification. 15 µl of 37 mg/ml KE (Aldrich) in 20% EtOH were added to a 250 µl fraction and the subsequent treatment was identical to that described above, except that the reaction was stopped with 0.5 M potassium borate (pH 7.0). The RNA was finally resuspended in 25 mM potassium borate.

Psoralen crosslinking of nuclear extract

Crosslinking of yeast nuclear extract was performed according to Wassarman (1993). The extracted RNA products were fractionated by electrophoresis on a 6% denaturing polyacrylamide gel and then subjected to Northern analysis (see above).

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References

Banroques,J. and Abelson,J.N. (1989) *Mol. Cell. Biol.*, **9**, 3710–3719.
Bindereif,A., Wolff,T. and Green,M.R. (1990) *EMBO J.*, **9**, 251–255.
Black,D.L. and Pinto,A.L. (1989) *Mol. Cell. Biol.*, **9**, 3350–3359.
Blencowe,B.J., Sproat,B.S., Ryder,U., Barabino,S. and Lamond,A.I. (1989) *Cell*, **59**, 531–539.
Bordonné,R., Banroques,J., Abelson,J. and Guthrie,C. (1990) *Genes Dev.*, **4**, 1185–1196.
Bringmann,P., Appel,B., Rinke,J., Reuter,R., Theissen,H. and Lührmann,R. (1984) *EMBO J.*, **3**, 1357–1363.
Brow,D.A. and Guthrie,C. (1988) *Nature*, **334**, 213–218.
Brown,J.D. and Beggs,J.D. (1992) *EMBO J.*, **11**, 3721–3729.
Cheng,S.C. and Abelson,J. (1987) *Genes Dev.*, **1**, 1014–1027.
Datta,B. and Weiner,A.M. (1991) *Nature*, **352**, 821–824.
Fabrizio,P., McPheeters,D.S. and Abelson,J. (1989) *Genes Dev.*, **3**, 2137–2150.
Fortner,D.M., Troy,R.G. and Brow,D.A. (1994) *Genes Dev.*, **8**, 221–233.
Frank,D. and Guthrie,C. (1992) *Genes Dev.*, **6**, 2112–2124.
Green,M.R. (1991) *Annu. Rev. Cell. Biol.*, **7**, 559–599.
Guthrie,C. and Patterson,B. (1988) *Annu. Rev. Genet.*, **22**, 387–419.
Hagenbüchle,O., Santer,M., Steitz,J.A. and Mans,R.J. (1978) *Cell*, **13**, 551–563.
Hamm,J. and Mattaj,I.W. (1989) *EMBO J.*, **8**, 4179–4187.

Hashimoto,C. and Steitz,J.A. (1984) *Nucleic Acids Res.*, **12**, 3283–3293.
Hausner,T.P., Giglio,L.M. and Weiner,A.M. (1990) *Genes Dev.*, **4**, 2146–2156.
Inoue,T. and Cech,T.R. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 648–652.
Kandels-Lewis,S. and Séraphin,B. (1993) *Science*, **262**, 2035–2039.
Lamond,A.I., Konarska,M.M., Grabowski,P.J. and Sharp,P.A. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 411–415.
Lesser,C.F. and Guthrie,C. (1993) *Science*, **262**, 1982–1988.
Li,Z. and Brow,D.A. (1993) *Nucleic Acids Res.*, **21**, 4645–4646.
Lin,R.J., Newman,A.J., Cheng,S.C. and Abelson,J. (1985) *J. Biol. Chem.*, **260**, 14780–14792.
Lossky,M., Anderson,G.J., Jackson,S.P. and Beggs,J. (1987) *Cell*, **51**, 1019–1026.
Madhani,H.D. and Guthrie,C. (1992) *Cell*, **71**, 803–817.
Madhani,H.D. and Guthrie,C. (1994) *Annu. Rev. Genet.*, **28**, 1–26.
Moazed,D. and Noller,H.F. (1989) *Nature*, **342**, 142–148.
Moazed,D., Stern,S. and Noller,H.F. (1986) *J. Mol. Biol.*, **187**, 399–416.
Moore,M.J., Query,C.C. and Sharp,P.A. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 303–358.
Nilsen,T.W. (1994) *Cell*, **78**, 1–4.
Parker,R., Siliciano,P.G. and Guthrie,C. (1987) *Cell*, **49**, 229–239.
Patterson,B. and Guthrie,C. (1987) *Cell*, **49**, 613–624.
Pikielny,C.W., Rymond,B.C. and Rosbash,M. (1986) *Nature*, **324**, 341–345.
Rinke,J., Appel,B., Digweed,M. and Lührmann,R. (1985) *J. Mol. Biol.*, **185**, 721–731.
Rymond,B. and Rosbash,M. (1992) In Jones,E.W., Pringle,J.R. and Broach,J.R. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 143–192.
Sawa,H. and Abelson,J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11269–11273.
Sawa,H. and Shimura,Y. (1992) *Genes Dev.*, **6**, 244–254.
Shannon,K.W. and Guthrie,C. (1991) *Genes Dev.*, **5**, 773–785.
Sharp,P.A. (1994) *Cell*, **77**, 805–815.
Siliciano,P.G., Brow,D.A., Roiha,H. and Guthrie,C. (1987) *Cell*, **50**, 585–592.
Sontheimer,E.J. and Steitz,J.A. (1993) *Science*, **262**, 1989–1996.
Steitz,J.A. (1992) *Science*, **257**, 888–889.
Vankan,P., McGuigan,C. and Mattaj,I.W. (1990) *EMBO J.*, **9**, 3397–3404.
Wassarman,D.A. (1993) *Mol. Biol. Rep.*, **17**, 143–151.
Wassarman,D.A. and Steitz,J.A. (1992) *Science*, **257**, 1918–1925.
Watkins,K.P. and Agabian,N. (1991) *Genes Dev.*, **5**, 1859–1869.
Wise,J.A. (1993) *Science*, **262**, 1978–1979.
Wise,J.A., Tollervey,D., Maloney,D., Swerdlow,H., Dunn,E.J. and Guthrie,C. (1983) *Cell*, **35**, 743–751.
Wolff,T. and Bindereif,A. (1992) *EMBO J.*, **11**, 345–359.
Wolff,T. and Bindereif,A. (1993) *Genes Dev.*, **7**, 1377–1389.
Wu,J.A. and Manley,J.L. (1991) *Nature*, **352**, 818–821.
Yean,S.L. and Lin,R.J. (1991) *Mol. Cell. Biol.*, **11**, 5571–5577.
Youvan,D.C. and Hearst,J.E. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 3751–3754.

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