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An unexpected, conserved element of the U3 snoRNA is required for Mpp10p association

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ABSTRACT

The U3 small nucleolar ribonucleoprotein (snoRNP) is composed of a small nucleolar RNA (snoRNA) and at least 10 proteins. The U3 snoRNA base pairs with the pre-rRNA to carry out the A0, A1, and A2 processing reactions that lead to the release of the 18S rRNA from the nascent pre-rRNA transcript. The yeast U3 snoRNA can be divided into a short 5′ domain (nt 1–39) and a larger 3′ domain (73 to the 3′ end) separated by a stretch of nucleotides called the hinge region (nt 40–72). The sequences required for pre-rRNA base pairing are found in the 5′ domain and hinge region whereas the 3′ domain is largely covered with proteins. Mpp10p, one of the protein components unique to the U3 snoRNP, plays a role in processing at the A1 and A2 sites. Because of its critical role in U3 snoRNP function, we determined which sequences in the U3 snoRNA are required for Mpp10p association. Unlike fibrillarin and all the previous U3 snoRNP components studied in this manner, sequences in the 3′ domain are not sufficient for Mpp10p association. Instead, a conserved sequence element in the U3 snoRNA hinge region is required, placing Mpp10p near the 5′ domain that carries out the pre-rRNA base-pairing interactions in the functional center of the U3 snoRNP.

Keywords: nucleolus; RNA processing; rRNA

INTRODUCTION

The nucleolus of eukaryotic cells contains a wide array of stable small nucleolar RNPs (snoRNPs) involved in processing of ribosomal RNA transcripts and in ribosome assembly (Kressler et al., 1999; Venema & Tollervey, 1999). The most abundant of the snoRNPs required for processing of pre-rRNA, the U3 snoRNP, has been studied in a number of different organisms. Functional studies on the role of the U3 snoRNA in mice, *Xenopus laevis*, or *Saccharomyces cerevisiae* have revealed that the U3 snoRNA is required for pre-18S rRNA processing (Kass et al., 1990; Savino & Gerbi, 1990; Hughes & Ares, 1991; Mougey et al., 1993; Borovjagin & Gerbi, 1999). In yeast cells, the U3 snoRNA base pairs with the pre-rRNA at two sites and is required for endonucleolytic cleavage at sites A0, A1, and A2 leading to 18S rRNA production (Hughes & Ares, 1991; Beltrame & Tollervey, 1992, 1995; Beltrame et al., 1994; Hughes, 1996; Sharma & Tollervey, 1999).

Comparison of the U3 snoRNAs among eukaryotic organisms has revealed several conserved sequence elements called boxes A, A′, B, C, C′, and D (Fig. 1A; Wise & Weiner, 1980; Jeppesen et al., 1988; Porter et al., 1988; Tyc & Steitz, 1989; Kiss & Solymosy, 1990; Myslinski et al., 1990; Hughes & Ares, 1991; Marshallsay et al., 1992; Tycowski et al., 1993). Secondary structure models for the U3 snoRNA have been proposed from a variety of U3 molecules based on computer folding, phylogenetic sequence comparison, and chemical and enzymatic mapping; yet, no consensus structure has emerged (Kiss et al., 1985; Hughes et al., 1987; Parker & Steitz, 1987; Jeppesen et al., 1988; Porter et al., 1988; Kiss & Solymosy, 1990; Myslinski et al., 1990; Marshallsay et al., 1992; Mazan et al., 1992; Segault et al., 1992; Selinger et al., 1992; Hartshorne & Agabian, 1994; Mereau et al., 1997). However, a common two-domain secondary structure model does exist for the yeast U3 snoRNA. In this model, a short 5′ domain (nt 1–39) is linked to a larger 3′ do-

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main (nt 73 to the 3′ end) via a sequence termed the hinge region (Fig. 1A; Samarsky & Fournier, 1998; Antal et al., 2000; Borovjagin & Gerbi, 2000).

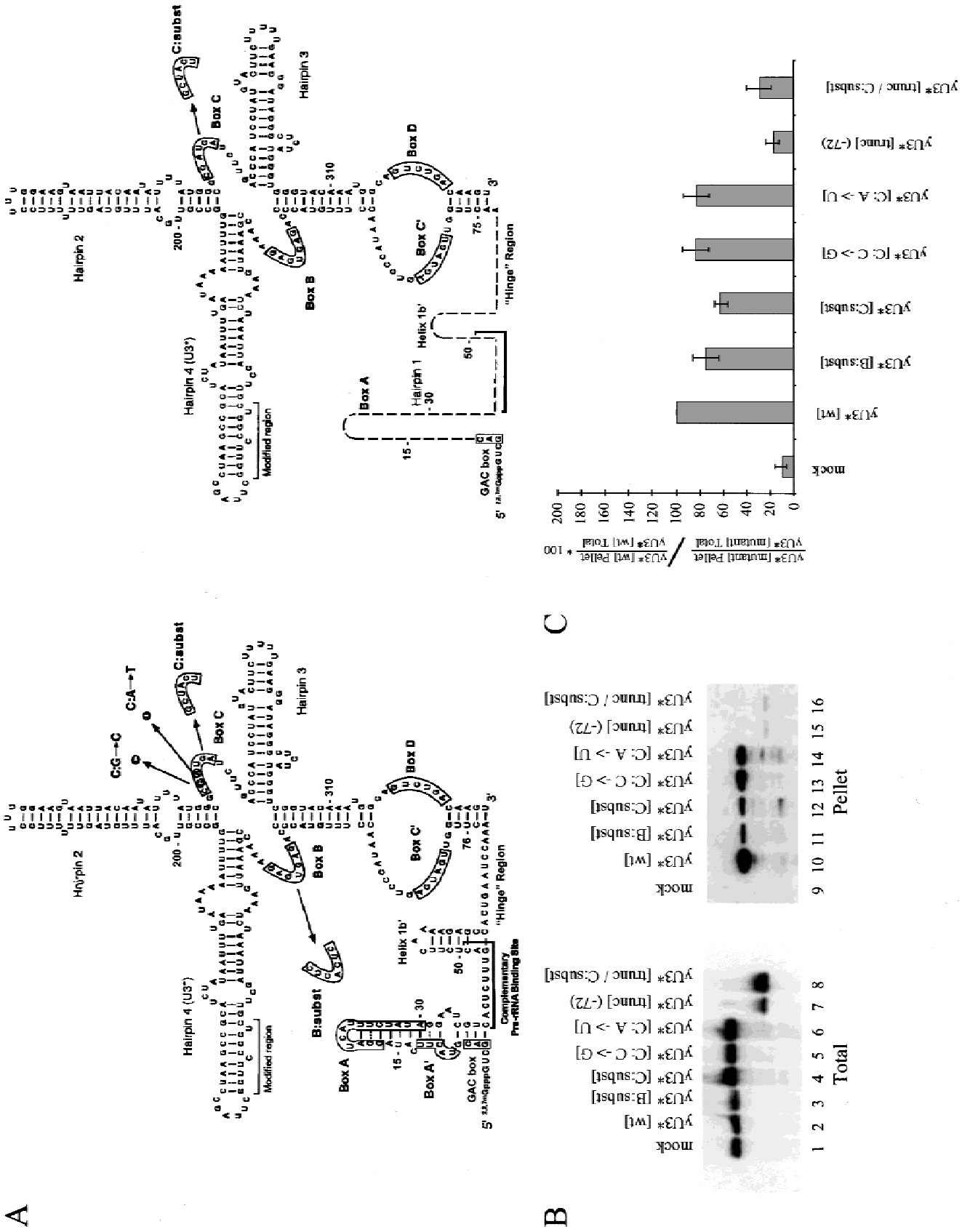
Two of the conserved box elements, boxes A and A′, can be found in the 5′ domain (Stroke & Weiner, 1989; Tyc & Steitz, 1989). One of these, box A, is essential for yeast cell viability based on both substitution and deletion mutations (Hughes, 1996; Mereau et al., 1997). Sequences in box A of the U3 snoRNA base pair with sequences in the pre-18S rRNA to carry out the cleavage reactions at A1/A2 (Hughes, 1996; Sharma & Tollervey, 1999). In addition, phylogenetic comparison of U3 snoRNA sequences has also revealed a novel GAC element 3 nt 3′ to the trimethylguanosine cap, which has been proposed to base pair with the pre-rRNA (Tyc & Steitz, 1992; Mereau et al., 1997; Samarsky & Fournier, 1998).

The 3′ domain of the U3 snoRNA is highly folded and complexed with proteins (Parker & Steitz, 1987; Jeppesen et al., 1988; Baserga et al., 1991; Lubben et al., 1993; Hartshorne & Agabian, 1994; Mereau et al., 1997; Speckmann et al., 1999). In all U3 molecules, boxes B/C and boxes C′/D are predicted to be in close proximity separated by phylogenetically conserved terminal and central stems. The remaining nonconserved hairpins of the U3 snoRNA in yeast are dispensable and are not required for U3 function or stability (Samarsky & Fournier, 1998). Structural probing of boxes B/C and boxes C′/D in trypanosomes, yeast, and human U3 snoRNPs revealed these boxes are protected and most likely covered with proteins (Parker & Steitz, 1987; Baserga et al., 1991; Hartshorne & Agabian, 1994; Mereau et al., 1997). In yeast, substitutions of the sequences comprising box B disrupt U3 function (Samarsky & Fournier, 1998). In *Xenopus*, substitution of these same sequences has no affect on function; however, substitution of sequences comprising box C affect function and nucleolar localization of the U3 snoRNA (Lange et al., 1998). Furthermore, mutations in box C affect fibrillarin association with U3 (Baserga et al., 1991; Lange et al., 1998; Samarsky & Fournier, 1998; Speckmann et al., 1999). Similarly, fibrillarin has been shown to bind directly to the box C/D sequence of the U16 snoRNA in *Xenopus* oocytes and in ooycte extracts (Fatica et al., 2000). Substitution of sequences in boxes C′ or D in yeast abolish U3 RNA levels, indicating that these sequences are required for U3 snoRNA accumulation (Samarsky & Fournier, 1998). Furthermore, mutations in box D and the 3′ terminal stem block cap formation and affect nucleolar localization and nuclear import in *Xenopus* (Baserga et al., 1992; Terns et al., 1995; Lange et al., 1998).

The hinge region, the spacing between the 5′ and 3′ domains of the U3 snoRNA, is one of the least understood segments of the U3 snoRNA. In yeast, the hinge region encompasses nt 39 to 72. A hairpin called helix 1b′ has been proposed to exist in snoRNPs in solution within these boundaries (Segault et al., 1992; Mereau et al., 1997). Helix 1b′ is sometimes drawn as part of a larger helix termed helix 1b or stem-loop 1b (Segault et al., 1992; Mereau et al., 1997; Antal et al., 2000). Although the primary sequence of the hinge region differs among eukaryotic organisms, its length is fairly well conserved (between 34 and 37 nt; Samarsky & Fournier, 1998). The 5′ portion of the hinge region (nt 39–48) base pairs with the pre-rRNA as a prerequisite for the cleavage reaction at A0 (Beltrame & Tollervey, 1992, 1995; Beltrame et al., 1994). Therefore portions of both the 5′ domain and the hinge region base pair with the pre-rRNA to effect pre-rRNA cleavage. Because of these RNA–RNA interactions, the two hairpins in the 5′ domain and hinge region of the U3 snoRNA are not likely to exist in vivo (Antal et al., 2000; Borovjagin & Gerbi, 2000).

The U3 snoRNA is complexed with both proteins common to box C/D snoRNPs and proteins specific for the U3 snoRNP. The common proteins, conserved from yeast to humans, are fibrillarin (Nop1p), Nop56p, Nop5/Nop58p, and Snu13p (Schimmang et al., 1989; Henriquez et al., 1990; Tollervey et al., 1991, 1993; Gautier et al., 1997; Wu et al., 1998; Lafontaine & Tollervey, 1999; Watkins et al., 2000). In addition, the U3 snoRNP contains at least seven other proteins that are not components of any other snoRNP—Sof1p, Mpp10p, Imp3p, Imp4p, Lcp5p, Rrp9p, and Dhr1p (Jansen et al., 1993; Dunbar et al., 1997; Wiederkehr et al., 1998; Wu et al., 1998; Lee & Baserga, 1999; Colley et al., 2000; Venema et al., 2000). Mpp10p, Imp3p, Imp4p, Rrp9p, and the common proteins have known metazoan homologs (Gautier et al., 1997; Pluk et al., 1998; Westendorf et al., 1998; Lee & Baserga, 1999; Lyman et al., 1999). In addition, Rcl1p is a protein that is associated with the U3 snoRNA, but is not considered to be a snoRNP component (Billy et al., 2000). All of the yeast U3 snoRNP proteins identified so far are essential for cell viability and for 18S rRNA production.

It is likely that the protein components specific to the U3 snoRNP play intrinsic roles in executing the U3 snoRNP-dependent pre-rRNA processing steps. Indeed, because no specific component is required for U3 snoRNA biogenesis or stability, their essential role in ribosome biogenesis must be distinct from maintenance of U3 snoRNA levels. Mutational analysis of the U3 snoRNP-specific protein, Mpp10p, indicates a precise role for this protein in pre-rRNA processing. Deletion of the carboxyl-terminus of Mpp10p leads to a defect in pre-rRNA cleavage at A1/A2, but not A0 (Lee & Baserga, 1997). This is identical to the processing defect observed in U3 snoRNAs that are mutated in the box A sequence. The mutations in these U3 snoRNAs disrupt one essential U3 snoRNA–pre-RNA base-pairing interaction (Hughes, 1996; Sharma & Tollervey, 1999). Taken together, these results suggest that Mpp10p may participate in maintenance of the U3 snoRNA–pre-


A
Hairpin 2
Hairpin 4 (U3*)
Modified region
Hairpin 3
Box C
C:subst
C:A→T
C:G→C
Box B
B:subst
Box A
Box A'
Box C'
Box D
Helix 1b'
Hairpin 1
GAC box
"Hinge" Region
Complementary Pre-rRNA Binding Site
B
mock
yU3* [wt]
yU3* [B:subst]
yU3* [C:subst]
yU3* [C: C -> G]
yU3* [C: A -> U]
yU3* [trunc] (-72)
yU3* [trunc / C:subst]
1 2 3 4 5 6 7 8
Total
9 10 11 12 13 14 15 16
Pellet
C
yU3* [mutant] Pellet / yU3* [mutant] Total
yU3* [wt] Pellet / yU3* [wt] Total * 100
0 20 40 60 80 100 120 140 160 180 200
mock
yU3* [wt]
yU3* [B:subst]
yU3* [C:subst]
yU3* [C: C -> G]
yU3* [C: A -> U]
yU3* [trunc] (-72)
yU3* [trunc / C:subst]

rRNA interaction in order to carry out the A1/A2 cleavage steps. Because of its critical role in U3 snoRNP function, we determined what sequences in the U3 snoRNA are required for Mpp10p association. We present evidence here that unlike all the previous U3 snoRNP components studied in this manner, sequences in the 3′ domain are not sufficient for Mpp10p association. Instead, a conserved sequence element in the U3 snoRNA hinge region is required, placing Mpp10p near the 5′ domain that carries out the pre-rRNA base-pairing interactions.

RESULTS AND DISCUSSION

The first 72 nt of the yeast U3 snoRNA are required for Mpp10 association

Using an in vivo system previously devised to test for function of mutated U3 snoRNAs in yeast, we have determined which sequences in the U3 snoRNA are required for association with both Mpp10p and fibrillarin (Nop1p). The central feature of this system is the use of a test strain, JH84, where one of the two genes encoding the U3 snoRNA (U3A) is disrupted with a *LEU2* marker gene and the second U3 gene (U3A) is under the control of a galactose inducible/glucose repressible promoter (Hughes & Ares, 1991; Samarsky & Fournier, 1998). U3 snoRNAs bearing mutations can tested by introduction of plasmid-borne U3 genes into this strain. The plasmid-encoded U3 snoRNA has been "tagged" with a unique nucleotide sequence to distinguish it from the wild-type U3 snoRNA in northern blots (termed yU3*). Provided the mutated snoRNAs accumulate, their ability to sustain growth and to associate with specific proteins can be tested by growth in glucose. The results that we have obtained on RNA accumulation, function, and Mpp10p and fibrillarin association that will be presented here are summarized in Table 1.

The initial series of experiments in this study were performed using several of the yeast U3* snoRNA mutations described in Samarsky & Fournier (1998). These yU3* alleles included mutations that separated the 5′ domain and hinge region of the yU3* molecule (nt 1–72; yU3* [trunc; −72]) from sequences in the 3′ domain (nt 73–333). In addition, mutations in the 3′ domain of U3 were used and included substitutions in the conserved box B (yU3* [B:subst]) and box C (yU3* [C:subst]) elements as well as point mutations in box C (yU3* [C:G → C] and yU3* [C:A → U])(Fig. 1A).

Immunoprecipitations were performed from whole-cell extracts of yeast strains bearing U3* snoRNA mutations with antibodies against yeast Mpp10p to determine which RNA sequences were required for their association. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blotting. For each trial of mutated yU3* snoRNAs, only one example gel is shown, but multiple, separate trials for each experiment were quantitated and are summarized. For quantitation, the ratio of mutated yU3* immunoprecipitated with Mpp10p antisera (pellet/total) to that of wild-type U3 immunoprecipitated with Mpp10p antisera (pellet/total) was calculated. The averages from several experiments are depicted in graphical format with error bars indicating standard error. In this way variability in RNA levels, and differences in immunoprecipitation and RNA extraction among different experimental trials, is minimized.

The results shown in Figure 1 indicate that sequences in the first 72 nt of the yeast U3 snoRNA, which includes the 5′ domain and hinge region, are required for Mpp10 association. Recovery of a 5′ truncated yU3* snoRNA [trunc; −72], bearing only nt 73 to the 3′ end of the U3 snoRNA, in the pellet of an anti-Mpp10p immunoprecipitate is greatly decreased when compared to the recovery of unmutated U3 snoRNA (Fig. 1B, lanes 15–16). This suggests that the 5′ domain and hinge sequences are required for Mpp10p association.

FIGURE 1 (*on facing page*)**.** Sequences in the first 72 nt of the U3 snoRNA are required for Mpp10p association. **A:** Proposed in vitro structure of the U3 snoRNA from the yeast *S. cerevisiae* (see text). The indicated mutations in the U3 snoRNA were tested for their ability to support Mpp10p association. The asterisk indicates a U3 snoRNA with a tag (see Materials and Methods; Samarsky & Fournier, 1998). These yU3* alleles include mutations that separate the 5′ domain and hinge region of the U3 snoRNA (nt 1–72) from sequences in the 3′ domain (nt 73 to end). The mutations in the 3′ domain of U3 included substitutions in the conserved box B (yU3* [B:subst]) and box C (yU3* [C:subst]) sequence elements as well as point mutations in box C (yU3* [C:G → C] and yU3* [C:A → U]). The mutations in the 5′ domain and hinge region included a deletion of the first 72 nt of U3 (yU3* [trunc; −72]) and a mutation where sequences in box C were substituted in this truncated U3 form (yU3* [trunc/C:subst]). **B:** Immunoprecipitation followed by northern blotting with a specific probe for the tagged U3 snoRNA. Immunoprecipitations were performed on yeast extracts expressing the mutated U3 snoRNAs with antibodies against yeast Mpp10p. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blots with an oligonucleotide (SD13*) that recognizes the hybridization tag found in the U3* snoRNAs. Total RNA represents 20% of that used for immunoprecipitation. **C:** Quantitation of the results presented in **B**. The ratio of mutated U3* immunoprecipitated with Mpp10p antisera (pellet/total) to that of wild-type U3 immunoprecipitated with Mpp10p antisera (pellet/total) was multiplied by 100 and the average result from three experiments is represented in graphical format. Error bars represent standard error. The results from the quantitation are: yU3* [B:subst], 74 ± 11.2%; yU3* [C:subst], 62 ± 5.4%; yU3* [C:G → C], 83 ± 10.9%; yU3* [C:A → U], 83 ± 11%; yU3* [trunc; −72], 18 ± 5.8%; and yU3* [trunc/C:subst], 29 ± 10.4%.

TABLE 1. Summary of the mutational analysis of the yeast U3 snoRNA.

U3 snoRNA mutations	RNA accumulation	Function	MPP10 association	Fibrillarin association
pRS313 (vector)[a]	−	−	−	−
yU3* (wt)[a]	+	+	+	+
yU3* [B:subst][a]	+	+ *cs*	+	−
yU3* [C:subst][a]	+	−	+	−
yU3* [C:G → C][a]	+	+	+	+
yU3* [C:A → T][a]	+	+	+	−
yU3* [C′:G → C][a]	−	−	nd	nd
yU3* [C′:A → T][a]	−	−	nd	nd
yU3* [D:A → T][a]	−	−	nd	nd
yU3* [trunc] [−72][a]	+	−	−	+
yU3* [trunc/C:subst][a]	+	−	−	−
yU3 [delet][a,b]	+	+	+[b]	+[b]
yU3* [GAC → CCU]	+	+	+	nd
yU3* [A′:subst]	+	+	+	nd
yU3* [A:subst]	+	−	+	nd
yU3* [JH125]	+	+ *cs*	+	nd
yU3 [JH137]	+	+ *cs*	+	nd
yU3* [−38]	+	−	+	nd
yU3* [−47]	+	−	+	nd
yU3* [−62]	+	−	−	nd
yU3* [1b′:delet]	+	−	−	nd
yU3* [1b′:P]	+	+	+	nd
yU3* [1b′:D]	+	+	+	nd
yU3* [1b′:PD]	+	+	+	nd
yU3* [1b′:loop]	+	+	+	nd
yU3* [A70][b]	−	−	nd	nd
yU3* [ACD][b]	−	−	nd	nd
yU3* [D6][b]	+	+	+[b]	nd
yU3* [P6C][b]	+	+	+[b]	nd

[a]Originally studied by Samarsky and Fournier (1998).
[b]Data not shown.
nd: not determined; *cs*: cold sensitivity.

Neither substitution of the conserved box B or box C elements nor mutations of box C affected Mpp10p association to the same extent (Fig. 1B, lanes 11–14). In addition, deletion of the three hairpins in the 3′ domain, which are not required for function, had no effect on Mpp10p association (Table 1; data not shown). The results were quantitated and are shown in Figure 1C.

The 3′ domain of the U3 snoRNA is required for fibrillarin association

For comparative purposes, immunoprecipitations were performed with extracts from the same strains with antibodies against yeast Nop1p (fibrillarin) to determine if different U3 snoRNA sequences were required for fibrillarin association. Immunoprecipitation with this anti-fibrillarin antibody leads to increased RNA degradation when compared to the anti-Mpp10p antibody, hence the presence of multiple bands on the gel. Because this is a comparison among different mutations in the U3 snoRNA, it does not interfere with interpretation of the results. In contrast to the anti-Mpp10 immunoprecipitations, truncation of the first 72 nt (the 5′ domain and hinge region) of the yeast U3* snoRNA had no effect on the recovery of U3 snoRNA (Fig. 2A, lane 15). This suggests that this portion of the U3 snoRNA is not required for fibrillarin–U3 association and that nt 73 to the 3′ end of the U3 snoRNA are sufficient for fibrillarin association. The recovery of U3 snoRNA with substitutions in box B or box C in the pellets of anti-fibrillarin immunoprecipitates was only slightly reduced when compared to wild type, about the same extent as with anti-Mpp10p association (Fig. 2A, lanes 10–12). When a single nucleotide in box C (G254C) is mutated, no effect is seen on fibrillarin association with the U3 snoRNA (Fig. 2A, lane 13). Interestingly, when a different nucleotide in box C (A255U) is mutated, fibrillarin association is again slightly reduced (Fig. 2A, lane 14). When the sequences in box C are substituted in the 5′-truncated U3* substrate, fibrillarin association is reduced to a greater extent than with the truncation alone (Fig. 2A, lane 16). The effect of mutations in boxes C′ and D on fibrillarin association could not be tested be-

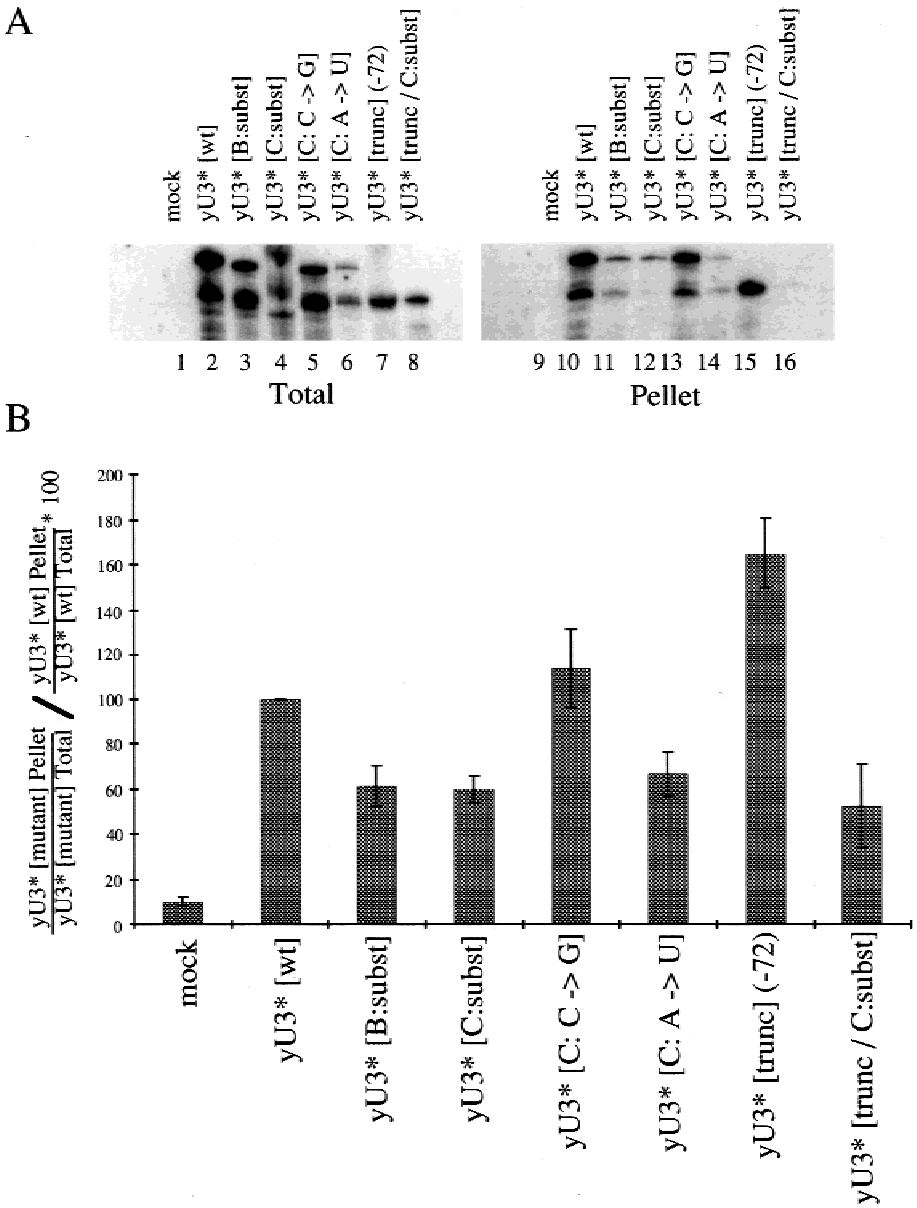


FIGURE 2. The 3′ domain (nt 73 to end) of the U3 snoRNA is required for fibrillarin association. For the mutations tested in this series of experiments, refer to Figure 1A. **A:** Immunoprecipitations were performed with antibodies against yeast fibrillarin (Nop1p) to determine which sequences of the tagged U3 snoRNA are required for fibrillarin association. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blots with an oligonucleotide (SD13*) that recognizes the hybridization tag found in the U3* RNAs. Total RNA represents 20% of that used for immunoprecipitation. **B:** Quantitation of results presented in **A**. Only the intact RNA (top band) in each lane was included in the quantitation. The ratio of mutated U3* immunoprecipitated with anti-fibrillarin antisera (pellet/total) to that of wild-type U3 immunoprecipitated with anti-fibrillarin antisera (pellet/total) was multiplied by 100 and the average result from three experiments is represented in graphical format. Error bars represent standard error. The results from the quantitation are: yU3* [B:subst], 61 ± 9.2%; yU3* [C:subst], 60 ± 5.8%; yU3* [C:G → C], 114 ± 17.4%; yU3* [C:A → U], 67 ± 9.6%; yU3* [trunc; −72], 165 ± 15.9%; and yU3* [trunc/C:subst], 52 ± 18.4%.

cause these RNA are not stable in vivo. Taken together, the truncation and mutation results indicate that sequences in the 3′ domain of the yeast U3 snoRNA are required for fibrillarin association. Importantly, these results indicate that diverse sequence elements in the U3 snoRNA are required for Mpp10p and for fibrillarin (Nop1p) association.

Sequences in the GAC element, box A′, or box A are not required for Mpp10p association

Because mutations in both Mpp10p and in a U3 snoRNA with mutations in the box A sequence share an interesting phenotype (Hughes & Ares, 1991; Lee & Baserga,

1997), we tested whether mutations in the box A sequence or adjacent sequences would affect Mpp10p-U3 snoRNA association. Immunoprecipitations with anti-Mpp10p antibodies were performed on extracts from strains expressing U3 snoRNAs with substitutions of the conserved box A′ (yU3* [box A′:subst]), box A (yU3* [box A:subst]) sequences elements, and the novel GAC element (yU3* [GAC → CCU]). Anti-Mpp10p immunoprecipitations were also performed on strains bearing U3 snoRNAs with previously described mutations in the box A sequence (yU3* [JH125] and yU3* [JH137]; Hughes, 1996). The mutations are depicted in Figure 3A. All the U3 snoRNAs bearing these various mutations accumulate in yeast (Fig. 3B, lanes 3–5 and 7–8). However, none of these mutations had any effect on Mpp10 association with the U3 snoRNA (Fig. 3B, lanes 11–13 and 15–16). As shown in Figure 2, the U3 snoRNA truncated to −72 did not associate with Mpp10p (lane 14).

Interestingly, despite the widespread conservation of the GAC element and box A′, neither is required for function (Table 1). However, substitution of the sequence in box A, which participates in one of the U3 snoRNA–pre-rRNA base-pairing interactions, impairs RNP function, as has been seen previously (Table 1; Hughes, 1996; Mereau et al., 1997).

Nucleotides 47 to 63 of yeast U3 snoRNA are required for Mpp10p association

The results from these two sets of analyses indicate that whereas sequences in the first 72 nt of the yeast U3 snoRNA are required for Mpp10p association, extensive mutations of the first 38 nt have no effect. Therefore, a series of deletion mutations in the 5′ domain and hinge region of the U3 snoRNA were created to determine which sequences in this region of U3 were required for Mpp10p association.

Sequential 5′ truncations of the U3 snoRNA were tested for their ability to associate with the Mpp10 protein. Deletions were made to −38 (yU3* [−38]), which deletes box A′ and box A sequences, and to −47 (yU3*[−47]), which deletes these sequences as well as the hinge sequences required for U3 snoRNA–pre-rRNA base pairing in the 5′ ETS (Fig. 4A). The mutated U3 snoRNAs produced in these strains accumulate in yeast (Fig. 4B, lanes 4 and 5). However, testing these mutant U3 snoRNAs revealed that recovery of the 5′-truncated U3 snoRNAs in the pellets of anti-Mpp10p immunoprecipitations was not affected (Fig. 4B, lanes 12 and 13). These results indicate that sequences in the first 47 nt of U3 are not required for Mpp10p association.

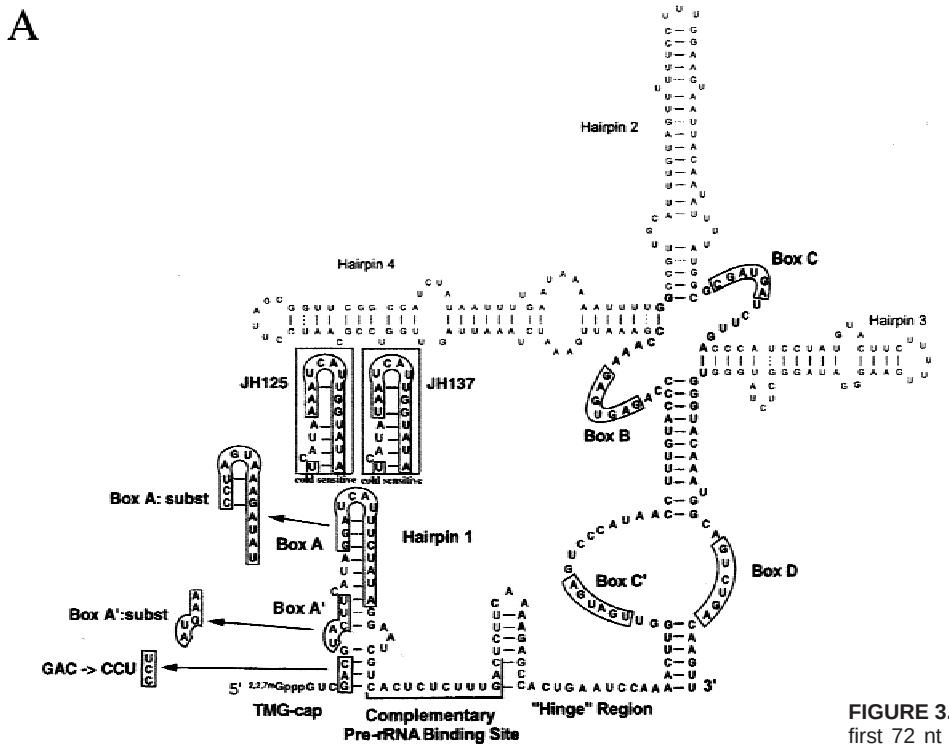
As expected, truncation of the U3* snoRNAs at the 5′ end does affect function (Table 1). This is likely due to the fact that these U3 snoRNAs lack the 5′ sequences necessary for interaction with the pre-rRNA.

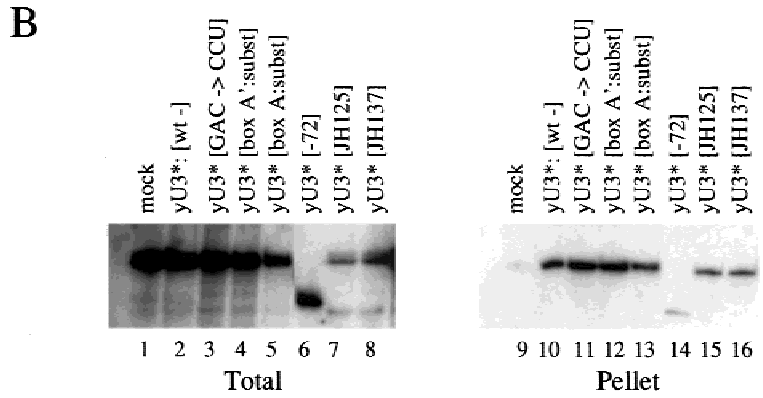
Because the first 72 nt of the U3 snoRNA were required for Mpp10p association but the first 47 nt were not, we reasoned that the sequences required for Mpp10p association were likely to reside between nt 47 and 72. Chemical and enzymatic mapping in the hinge region of U3 in *S. cerevisiae* indicated the possible existence of a small hairpin loop termed helix 1b′ within the hinge sequence (Segault et al., 1992; Mereau et al., 1997). Helix 1b′ consists of 13 nt in the hinge region of the U3 snoRNA, between the stem containing boxes A′ and box A and the stem containing boxes B, C, C′, and D (Fig. 4A). A 5′ truncation was made in the U3 snoRNA where the sequences, which comprise helix 1b′, were removed. In this U3 snoRNA, the GAC element, box A′, and box A sequences were deleted in addition to the sequences corresponding to helix 1b′ (nt 47–63; yU3* [−63]). RNA produced from this mutated U3* snoRNA allele is stable in cells (Fig. 4B, lane 6). Immunoprecipitations with anti-Mpp10p antibodies indicated that the deletion of these sequences greatly reduced the recovery of the mutated U3 snoRNAs in the immunoprecipitate (Fig. 4B, lane 14). This suggests that nt 47–63, comprising the proposed helix 1b′ of the U3 snoRNA, are required for Mpp10p association. This U3* allele does not support growth on media containing glucose (Table 1).

Helix 1b′ tolerates extensive mutations without disrupting Mpp10p association

Because 5′ truncation of sequences, which include helix 1b′, reduced Mpp10p association with the U3 snoRNA, we reasoned that specific structural elements within this helix might be required for Mpp10p association. To investigate this, five mutations were made in helix 1b′ (Fig. 5A). In these U3* snoRNAs, the sequences in the 5′ domain and hinge region of U3 were restored and only the sequences comprising helix 1b′ were deleted or mutated. In yU3* [1b′:delet] the entire stem-loop containing helix 1b′ was deleted in the context of the U3 snoRNA. In yU3* [1b′:P] and yU3* [1b′:D] the proximal 5 nt of helix and the distal 5 nt of helix 1b′ were substituted, respectively. These mutations led to disruption of the stem that forms helix 1b′. A mutation was made in yU3* [1b′:P] that restores the stem by making compensatory substitutions in both (yU3* [1b′:PD]). This mutation restores the structure of helix 1b′ yet reverses the sequences that comprise the stem. An additional mutation was made to investigate whether the sequences in the loop of helix 1b′ were required for Mpp10p association (yU3* [1b′:LP]). These mutated U3 snoRNAs accumulated in cells (Fig. 5B, lanes 2–6).

Anti-Mpp10p immunoprecipitations were performed from yeast extracts made from strains expressing the U3 snoRNAs with mutations in helix 1b′ to determine which sequences are required for Mpp10p association.





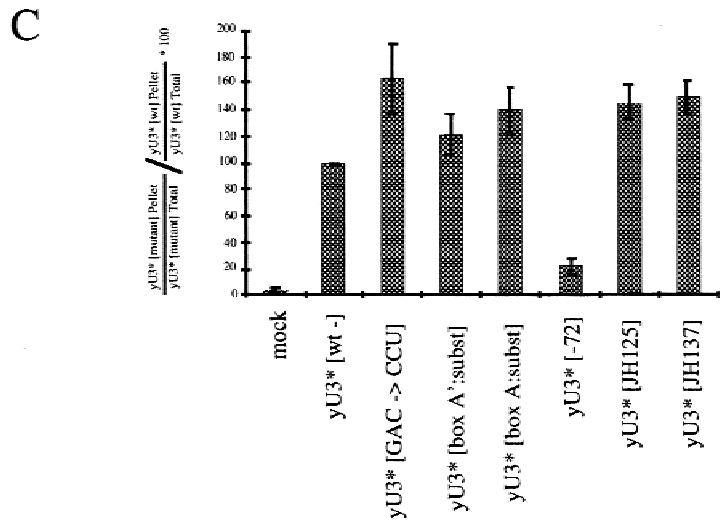


FIGURE 3. Conserved sequence elements in the first 72 nt of the U3 snoRNA are not required for Mpp10p association. **A:** Mutations were made in the conserved GAC element (yU3* [GAC → CCU]), box A′ (yU3* [box A′: subst]), or box A (yU3* [box A: subst]) of the yeast U3 snoRNA. Two previously characterized nucleotide substitutions in the U3 box A element, JH125 and JH137, were also examined (Hughes, 1996). In addition, a deletion of the entire stem containing the conserved GAC element, box A′, and box A was studied (yU3* [−38]). **B:** Immunoprecipitations were performed from extracts harboring these mutated U3 snoRNAs with antibodies against yeast Mpp10p to determine which sequences in the 5′ domain or hinge region of the tagged U3 snoRNA are required for Mpp10p association. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blots with an oligonucleotide (SD13*) that recognizes the hybridization tag found in the U3* RNAs. Total RNA represents 20% of that used for immunoprecipitation. **C:** Quantitation of results presented in **B**. The ratio of mutated U3* immunoprecipitated with Mpp10p antisera (pellet/total) to that of wild-type U3 immunoprecipitated with Mpp10p antisera (pellet/total) was multiplied by 100 and the average result from six experiments is represented in graphical format. Error bars represent standard error. The results from the quantitation are: yU3* [GAC → CCU], 163 ± 26.3%; yU3* [box A′:subst], 120 ± 15.4%; yU3* [box A:subst], 131 ± 18.1%; yU3* [trunc; −72], 22 ± 6.0%; yU3* [JH125], 146 ± 12.5%, and yU3* [JH137], 150 ± 12.1%.

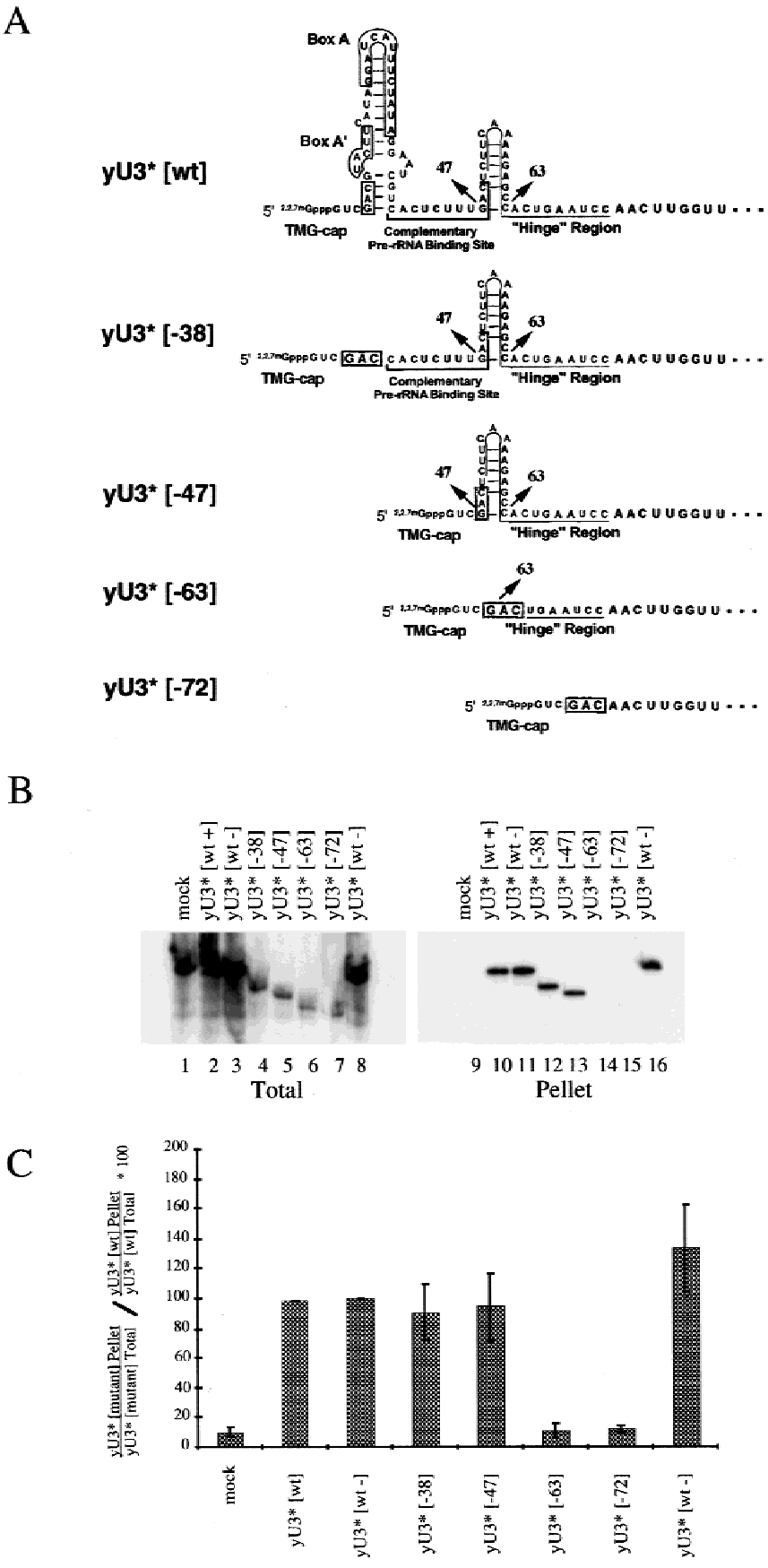


FIGURE 4. Nucleotides 47–63 of the U3 snoRNA are required for Mpp10p association. **A:** A series of deletion mutants were made to pinpoint the sequences in the 5′ domain or hinge region of the yeast U3 snoRNA that are required for Mpp10p association. Two additional deletions were made in addition to the deletion of the entire hairpin containing the conserved GAC element, box A′, and box A (yU3* [−38]). One is a deletion that removed this hairpin in addition to sequences in the hinge region that interact with pre-rRNA (yU3* [−47]). The second deletion encompasses the previous mutation and removes sequences which form helix 1b′ in the hinge region of U3 (yU3* [−67]). The full-length U3 snoRNA with an intron (yU3* [wt −]) and without an intron (yU3* [wt −] as well as the truncated U3 snoRNA mutation (yU3* [trunc; −72]) were included. **B:** Immunoprecipitations were performed with antibodies against yeast Mpp10p to determine which sequences of the U3* snoRNA are required for Mpp10p association. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blots with an oligonucleotide (SD13*) that recognizes the hybridization tag found in the U3* RNAs. Total RNA represents 20% of that used for immunoprecipitation. **C:** Quantitation of results presented in **B**. The ratio of mutated U3* immunoprecipitated with Mpp10p antisera (pellet/total) to that of wild-type U3 immunoprecipitated with Mpp10p antisera (pellet/total) was multiplied by 100 and the average result from five experiments is represented in graphical format. Error bars represent standard error. The results obtained upon quantitation are: yU3* [−38], 91 ± 18.4%; yU3* [−47], 95 ± 22.8%; yU3* [−63], 10 ± 4.7%; yU3* [trunc; −72], 12 ± 2.32%; and yU3* [wt −], 134 ± 29.2%.

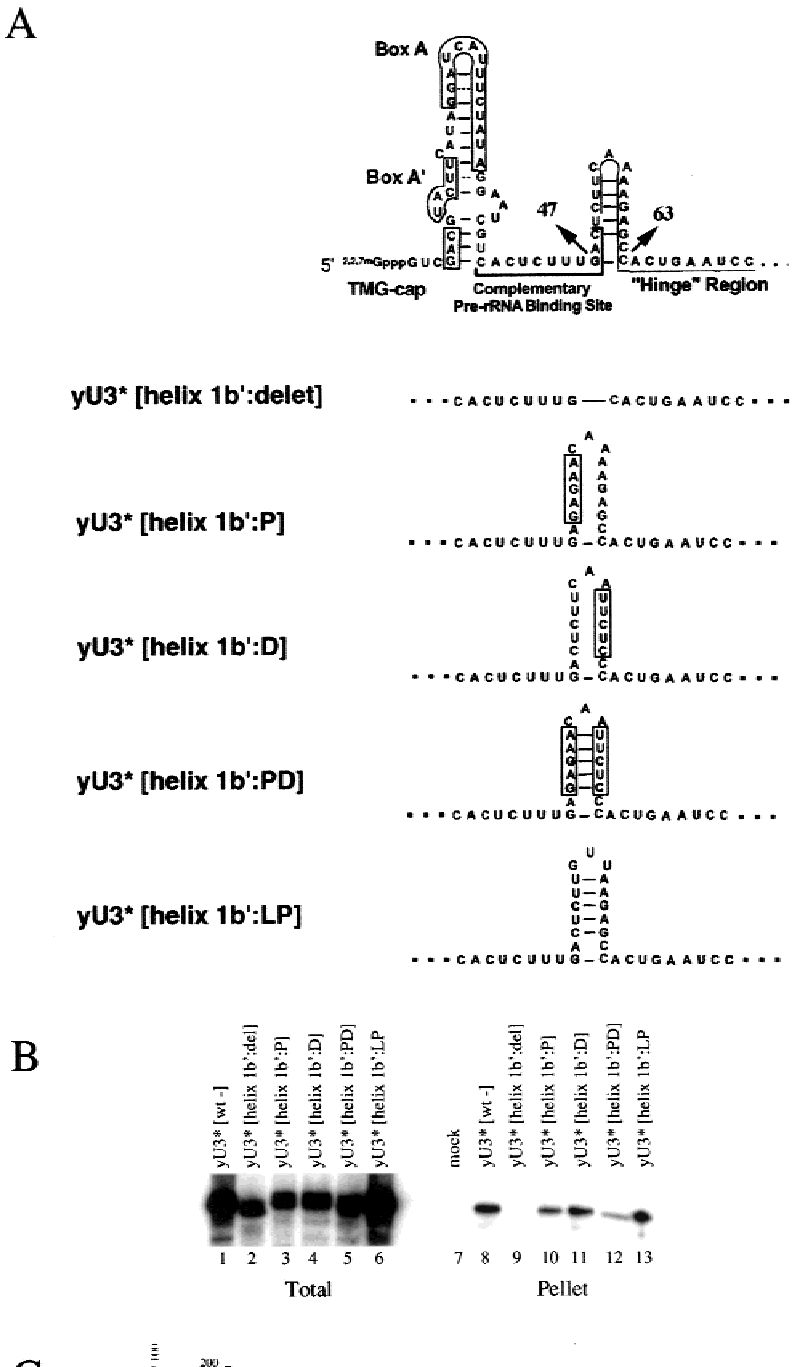


FIGURE 5. Helix 1b′ tolerates extensive mutation before Mpp10p association is lost. **A:** In addition to a deletion that removes only the sequences of helix 1b′ (yU3* [helix 1b′: delet]), a series of substitution mutations were made in the sequences of helix 1b′. The proximal (yU3* [helix 1b′:P]) and distal (yU3* [helix 1b′:D]) 5 nt that form the stem were substituted, independently. Then, these substitution mutations were combined to restore the base pairing in the stem by reversing the nucleotides that comprise the stem (yU3* [helix 1b′:PD]). Finally, a mutation was made in the sequences that comprise the loop of helix 1b′ (yU3* [helix 1b′: LP]). **B:** Immunoprecipitations were performed with antibodies against yeast Mpp10p on extracts from yeast expressing the U3 snoRNAs described in **A**. RNA was extracted from the pellets and analyzed by gel electrophoresis and northern blots with an oligonucleotide (SD13*) that recognizes the hybridization tag found in the U3* RNAs. Total RNA represents 20% of that used for immunoprecipitation. **C:** Quantitation of results presented in **B**. The ratio of mutated U3* immunoprecipitated with Mpp10p antisera (pellet/total) to that of wild-type U3 immunoprecipitated with Mpp10p antisera (pellet/total) was multiplied by 100 and the average result from five experiments is represented in graphical format. Error bars represent standard error. The results from the quantitation are: yU3* [1b′:delet]), 24 ± 6.4%; yU3* [1b′:P], 168 ± 12.6%; yU3* [1b′:D], 185 ± 17.6%; yU3* [1b′:delet], 130 ± 21%; and yU3* [1b′:LP], 113 ± 24.9%.

As expected, deletion of the entire stem-loop comprising helix 1b′ in the context of the U3 snoRNA greatly reduced Mpp10p association when compared to wild type (Fig. 5B, lane 9). Yet, when the sequences that comprise the proximal or distal stem of helix 1b′ are substituted (yU3* [1b′:P] and yU3* [1b′:D]), no effect was seen upon Mpp10p association (Fig. 5B, lanes 10–11). In addition, neither substitution of the sequences on both the proximal and distal stem of helix 1b′ (yU3* [helix 1b′:PD]) nor substitution of the sequences in the loop of helix 1b′ affected Mpp10p association (Fig. 5B, lanes 12 and 13). Taken together, these results indicate that although nt 47–63 are required for Mpp10p association, these nucleotides can tolerate extensive mutations before association is disrupted.

Interestingly, of these mutated U3 snoRNAs, only yU3* [1b′:delet], which deletes the entire 13 nt of helix 1b′, does not support growth (Table 1). Therefore, for these mutations in helix 1b′, defects in Mpp10p association and U3 snoRNP function were correlated. This is consistent with the role of Mpp10p as an essential component required for the function of the U3 snoRNP in pre-rRNA processing.

It has been proposed that the hinge sequence, which includes helix 1b′, serves as a spacer sequence between the two domains of the U3 snoRNP (Borovjagin & Gerbi, 2000). We considered the possibility that deletion of helix 1b′ in the context of the U3 snoRNA interfered with maintenance of a required distance between the 5′ and 3′ domains. To assess this further, we constructed two mutated U3 snoRNA bearing either an insertion or deletion in the hinge sequence. One, yU3*[D6], bears a deletion of nt 52–57. The other, yU3*[P6C], bears an insertion of six cytidines at nt 51. Neither mutation affected Mpp10p association nor U3 snoRNP function (Table 1; data not shown).

The mutational analysis of the U3 snoRNA described here suggests that nt 47–63 of the U3 snoRNA are required for Mpp10p association in the context of the U3 snoRNA. Because the 3′ domain (nt 73 to the 3′ end) of the U3 snoRNA was not sufficient for Mpp10p association, we designed mutated U3 snoRNAs to ask whether the 5′ domain plus the hinge region of the U3 snoRNA, including nt 47–63, were sufficient for Mpp10p association. Two RNAs were tested. The first consisted of only nt 1–70 of the U3 snoRNA yU3 [A70]. Unfortunately, it did not accumulate in yeast cells (Table 1; data not shown). This is not unexpected because intact boxes C′ and D are required for U3 snoRNA accumulation (Samarsky & Fournier, 1998). In an attempt to stabilize it, we placed a minimal box C and D sequence at nt 70 (yU3 [ACD]) (Xia et al., 1997; Watkins et al., 1998). Again, this U3 snoRNA did not accumulate in yeast cells (Table 1; data not shown). We were therefore unable to test whether sequences in the 5′ domain and the hinge region were sufficient for Mpp10p association.

In summary, utilizing an in vivo genetic system, we determined which nucleotides of the U3 snoRNA were required for association with two protein components of the U3 snoRNP, Mpp10p, and fibrillarin (Nop1p). By creating a series of mutations in the yeast U3 snoRNA that do not affect snoRNA stability, we determined that nucleotides in the hinge region, specifically nt 47 to 63, which comprise a structure found in solution termed helix 1b′, were required for association with Mpp10p. Neither substitution of the conserved box A′ and box A elements in the 5′ domain nor a series of 5′ deletions to nt 47 disrupted the formation of a U3 snoRNP containing Mpp10p. This is the first demonstration that sequences outside of the 3′ domain of the U3 snoRNA are required for association of a U3 snoRNP-specific protein. In contrast to our results with anti-Mpp10p association, the 3′ domain of the U3 snoRNA (nt 73 to the 3′ end) was sufficient for fibrillarin association. The sequences in the U3 snoRNA required for Mpp10p and for fibrillarin association are therefore distinct.

We did not expect to find that helix 1b′ (nt 47–63) was required for Mpp10p association. Previous results from studies in several different organisms have implicated only the 3′ domain (nt 73 the 3′ end) of the U3 snoRNA in protein binding (Parker & Steitz, 1987; Jeppesen et al., 1988; Segault et al., 1992; Hartshorne & Agabian, 1994; Mereau et al., 1997). Indeed, the 3′ domain of the U3 snoRNA is sufficient for association of the common proteins, Nop56, Nop5/58, fibrillarin, and Snu13p as well as the specific protein, Rrp9p (Lubben et al., 1993; Pluk et al., 1998; Lukowiak et al., 2000; Venema et al., 2000; Watkins et al., 2000). Because the stem on the distal side of helix 1b′ is protected during chemical and enzymatic mapping in vivo (Segault et al., 1992; Mereau et al., 1997), it has been proposed that helix 1b′ may form an additional base-pairing interaction with the pre-rRNA. One possible interpretation of our results is that this interaction is required for Mpp10p association and for overall U3 snoRNP function. Alternatively, these nucleotides in the U3 snoRNP are protected because Mpp10p and perhaps other proteins are situated there.

Because Mpp10p is found in yeast and metazoans, if the sequence constituting helix 1b′ is truly important for Mpp10p association it would be conserved among different species. Indeed, phylogenetic comparison of hinge region sequences indicates the capability to form a similar hairpin in the majority of organisms whose sequence is known. In mammalian U3 snoRNAs, multiple hairpins are even possible. Of the 20 sequenced U3 snoRNAs from different species, there are only four U3 snoRNAs where no hairpin can be drawn, those from *Dictyostelium discoideum*, *Schizosaccharomyces pombe*, *Xenopus laevis*, and *Xenopus borealis*. In general, the sequences that comprise the stem of helix 1b′ are also highly conserved. For example, two hairpins representing potential helix 1b′ structures can be drawn

for the human U3 snoRNA, although the length of the loop differs from that of yeast. However, although both stem-loop structures in the 5′ domain and the hinge region are well conserved, it is thought that they do not form in vivo when the U3 snoRNA is base paired with the pre-rRNA (Mereau et al., 1997; Antal et al., 2000). Consistent with this is the high level of tolerance of Mpp10p association to mutation of the involved sequences, even to the point of eliminating the helix 1b′ structure. Conservation, then, of the sequences required for Mpp10p association is likely due to a functional requirement other than maintenance of the structure of helix 1b′.

The hinge sequence, which includes helix 1b′, has been proposed to serve as a spacing sequence between the two domains of the U3 snoRNA (Antal et al., 2000; Borovjagin & Gerbi, 2000). Supporting this, mutations in the helix 1b′ analogous U3 snoRNA sequences of *X. laevis* did not lead to defects in pre-rRNA processing unless the length of this sequence was increased or decreased. This is very similar to the results that we have obtained by mutagenizing the *S. cerevisiae* U3 snoRNA, although we have found that certain deletions/insertions may be too small to cause an effect on function and Mpp10p association. If spacing between the 5′ and 3′ domains (domains I and II in *Xenopus*) is essential for U3 snoRNP function, perhaps it is because these sequences are required for association of specific proteins, like Mpp10p, required for pre-rRNA processing.

Although it is clear that nucleotides in the hinge region of the U3 snoRNA are required for Mpp10p association, this does not necessarily mean that these nucleotides are the binding site for Mpp10p. First, because these experiments were carried out in vivo, additional proteins are also present. Because of this, another protein or proteins may be interposed between Mpp10p and the U3 snoRNA. Unfortunately, direct binding studies have not yet been carried out because we have been unable to express Mpp10p in a soluble form in *Escherichia coli*. Second, loss of Mpp10p association may also be attributable to changes in RNA structure caused by deletion of helix 1b′. Disruption of this conserved hairpin may result in misfolding of the U3 snoRNA, leading to an RNA structure that does not support Mpp10p association. If so, it is still very specific: large deletions of other portions of the U3 snoRNA also likely to change RNA structure did not lead to loss of Mpp10p association. Our results are therefore consistent with the U3 snoRNA attaining a particular tertiary structure that is required for Mpp10p association.

Our results place Mpp10p in the functional center of the U3 snoRNP, consistent with previous genetic analyses (Lee & Baserga, 1997). Mpp10p interacts with two other U3 snoRNP-specific proteins, Imp3p and Imp4p. We do not yet know if Mpp10p or one of its interacting proteins contact the U3 snoRNA directly. Future studies will aim to verify the organization of Mpp10p, Imp3p, and Imp4p in the U3 snoRNP and to elucidate their topology with respect to the U3 snoRNA.

MATERIALS AND METHODS

Strains and media

The *S. cerevisiae* strain *JH84* (*α leu2-3,12 ura3-52 his3-*Δ *ade2-1 can1100 u3a*Δ *UAS$_{GAL}$:U3A::URA3 U3B::LEU2*) was used as the test strain for these experiments (Hughes & Ares, 1991; Samarsky & Fournier, 1998). Yeast cells were grown at 30 °C unless otherwise specified. Plasmids (*Amp^R, HIS3, CEN/ARS*) containing modified U3 sequences were introduced into the yeast strain JH84 by lithium acetate transformation (Gietz et al., 1995). Transformants were grown on selective solid 0.67% yeast nitrogen base (YNB) medium containing 2% galactose lacking histidine. All plasmids used in this study are listed in Table 2.

To test whether the mutated U3 snoRNAs were competent for growth, functional mapping of the mutated U3 genes was carried out as described in Samarsky and Fournier (1998). Briefly, transformants were restreaked from galactose minus histidine plates onto plates containing 5% glucose minus histidine. Cells were restreaked onto fresh glucose minus histidine plates and incubated for another 5-day period to confirm the results. Cold sensitivity of all of the new U3 snoRNA alleles was assayed by growth at 16 °C.

Preparation of modified U3 coding sequences

The plasmid yU3* contains a copy of a tagged wild-type U3 snoRNA gene in the yeast expression vector pRS313 (*Amp^R, HIS3, CEN/ARS*) (called yU3*.313 in Samarsky & Fournier, 1998; Sikorski & Hieter, 1989). Plasmids used for yeast transformation were prepared by removing the *Sal*I-*Eco*RI fragment found in yU3* and cloning *Sal*I-*Eco*RI PCR fragments carrying wild-type and mutated U3* RNA genes inserted into the appropriate restriction sites in yU3*.313. Restriction endonucleases used for verifying the size of the final products were *Sal*I and *Eco*RI. The oligonucleotides used for PCR mutagenesis are found in Table 3. PCR mutagenesis was performed using the universal M13 forward (5′-GTAAAAC GACGGCCAGT-3′) primer in conjunction with the specific oligonucleotide primers designated in Table 3.

The tested plasmids encoding mutated U3 snoRNAs are listed in Table 2 and include: yU3* [wt −], tagged U3* minus intron; yU3* [GAC → CCU], substitution of the conserved GAC element with the nucleotides CCU; yU3* [box A′: subst], substitution of the entire box A′ sequence UACUU with AUGAA; yU3* [box A: subst], substitution of the entire box A sequence GGAUCAUUUCUAUA with CCUAGUAAAGAUAU; yU3* [−38], deletion of the first 38 nt of the U3 snoRNA that contain boxes A′ and box A; yU3* [−47], deletion of the first 47 nt of the U3 snoRNA that removes boxes A′, box A, and sequences responsible for interaction with pre-rRNA; yU3* [helix 1b′: delet], deletion of nt 47–63 of the yeast U3 snoRNA which removes a potential hairpin termed helix 1b′; yU3* [helix 1b′: P], substitution of the proximal sequence CUCUU

TABLE 2. Plasmids used in this study.

Plasmids	Comments	Reference
pRS313	Plasmid vector with no insert	Samarsky and Fournier, 1998
pRU3	Wild-type U3	Samarsky and Fournier, 1998
pRU3*	Tagged U3*	Samarsky and Fournier, 1998
pRU3* [B:subst]	Substitution of the entire box B sequence GAGUGAG with CUCACUC	Samarsky and Fournier, 1998
pRU3* [C:subst]	Substitution of the entire box C sequence CGAUGA with GCUACU	Samarsky and Fournier, 1998
pRU3* [C:G → C]	Substitution of the first G in box C with C	Samarsky and Fournier, 1998
pRU3* [C:A → U]	Substitution of the first A in box C with U	Samarsky and Fournier, 1998
pRU3* [trunc/−72]	Deletion from the 5′ end to the base of the terminal stem	Samarsky and Fournier, 1998
pRU3* [trunc/C:subst]	Deletion from the 5′ end to the base of the terminal stem and substitution of the entire box C sequence CGAUGA with GCUACU	Samarsky and Fournier, 1998
pRU3 [delet]	Deletion of hairpins 2, 3, and 4 of the 3′ domain of the U3 snoRNA	Samarsky and Fournier, 1998
pRU3* [wt −]	Tagged U3* minus intron	This study
pRU3* [GAC → CCU]	Substitution of the conserved GAC element with the nucleotides CCU	This study
pRU3* [box A′: subst]	Substitution of the entire box A′ sequence UACUU with AUGAA	This study
pRU3* [box A: subst]	Substitution of the entire box A sequence GGAUCAUUUCUAUA with CCUAGUAAAGAUAU	This study
pRU3* [JH125]	Four base substitution of the box A sequence GGAUCAUUUCUAUA with AAAUCAUUGGUAUA	Hughes, 1991; this study
pRU3* [JH137]	Four base substitution of the box A sequence GGAUCAUUUCUAUA with UAAUCAUUGGUAUA	Hughes, 1991; this study
pRU3* [−38]	Deletion of the first 38 nt of the U3 snoRNA, which contain boxes A′ and box A	This study
pRU3* [−47]	Deletion of the first 47 nt of the U3 snoRNA, which removes box A′, box A, and sequences responsible for interaction with pre-rRNA	This study
pRU3* [helix 1b′: delet]	Deletion of nucleotides 47 to 63 of the yeast U3 snoRNA which removes a potential hairpin termed helix 1b′	This study
pRU3* [helix 1b′: P]	Substitution of the proximal sequence CUCUU of helix 1b′ with GAGAA	This study
pRU3* [helix 1b′: D]	Substitution of the distal sequence GAGAA of helix 1b′ with CUCUU	This study
pRU3* [helix 1b′: PD]	Substitution of both the proximal and distal sequences of helix 1b′	This study
pRU3* [helix 1b′: LP]	Substitution of the sequence CAA in the loop of helix 1b′ with GUU	This study
pRU3 [A70]	Deletion of the 3′ region of U3 leaving only the first 70 nt of U3	This study
pRU3 [ACD]	Substitution of the 3′ region of U3 with a minimal U14 box C and box D sequence	This study
pRU3 [D6]	Deletion of the nucleotides UCUUCA (52–57) of helix 1b′	This study
pRU3 [P6C]	Insertion of the nucleotides CCCCCC at position 51 prior to helix 1b′	This study

of helix 1b′ with GAGAA; yU3* [helix 1b′: D], substitution of the distal sequence GAGAA of helix 1b′ with CUCUU; yU3* [helix 1b′: PD], substitution of both the proximal and distal sequences of helix 1b′; and yU3* [helix 1b′: LP], substitution of the sequence CAA in the loop of helix 1b′ with GUU.

The mutations made by Samarsky and Fournier (1998) were made in the naturally occurring U3 gene, which contains an intron. All new mutations made here were made in an intronless cassette for ease of construction. Deletion of the intron in the U3 snoRNA precursor (yU3* [wt −] does not affect U3 snoRNA levels (Fig. 5B, lane 3) or growth in yeast (Table 1).

The sequences of the final inserts were verified by automated sequencing using an Applied Biosystems 373 Stretch sequencer at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. The oligonucleotide used to verify sequences was T3ASP (5′-ACCCTCACTAAAGGG AACAA-3′), which recognizes sequences in the T3 promoter found upstream of the cloned inserts in the yeast expression vector pRS313 (Sikorski & Hieter, 1989).

Antibodies

The anti-fibrillarin antibody 17C12 was obtained from Michael Pollard, Scripps Research Institute (Balakin et al., 1996). Antibodies to the yeast Mpp10p protein were obtained by injection into rabbits, as previously described (Dunbar et al., 1997).

Immunoprecipitations

For immunoprecipitations, antibodies were bound to 2.5 mg Protein A-Sepharose (PAS) CL-4B (Pharmacia) as follows: anti-Mpp10 (50 μL of rabbit serum) or anti-fibrillarin (100 μL of culture supernatant) for 16 h at 4 °C, rotating. The bound antibodies were washed three times with NET-2 (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.1% NP-40). A mock immunoprecipitation with no added antibody (PAS CL-4B alone) was also done. All immunoprecipitations and washing of the immunoprecipitates were performed in NET-2 buffer.

For preparation of yeast cell lysates, exponentially growing cells were harvested between OD_{600} 0.2–0.5, washed twice

TABLE 3. Oligonucleotides used in this study.

Mutation	Oligo	Sequence
Probe	SD13*	5′-GCGGCTTAGGCTAAGCTAAGGCCAGC-3′
Probe	SD74	5′-ATCAAGATCATCGCGCCGGTTTCTCACTCTGGGGTAC-3′
PCR	M13FOR	5′-GTAAAACGACGGCCAGT-3′
PCR	M13REV	5′-GGAAACAGCTATGACCATG-3′
Sequencing	T3ASP	5′-ACCCTCACTAAAGGGAACAA-3′
yU3* [wt]	YU3WT	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTC-3′
yU3* [GAC → CCU]	YU3GACD	5′-GAAGGCCTGTACTTCATAGGATCATTTC-3′
yU3* [Box A′: subst]	YU3APSUB	5′-TGACTCTGTCGACGATGAACATAGGATCATTTCTATAGGAATCGTC-3′
yU3* [Box A: subst]	YU3ASUB	5′-TGACTCTGTCGACGTACTTCAATCCTAGTAAAGATATCGAATCGTCACTCTTTGACTCTTC AAAAGAGCCACTG-3′
yU3* [JH125]	YU3JH125	5′-TGACTCTGTCGACGTACTTCATAAAATCATTGGTATAGGAATCGTCACTCTTTTGACTC-3′
yU3* [JH137]	yU3JH137	5′-TGACTCTGTCGACGTACTTCATATAATCATTGGTATAGGAATCGTCACTCTTTTGACTC-3′
yU3* [−38]	YU3ADEL	5′-TGACTCTGTCGACCACTCTTTGACTCTTCAAAAGAGCC-3′
yU3* [−47]	YU350	5′-TGACTCTGTCGACTCTTCAAAAGAGCCACTG-3′
yU3* [−63]	YU365	5′-TGACTCTGTCGACTGAATCCAACTTGGTTGATG-3′
yU3* [helix 1b′: delet]	YU3BDEL	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGCACTGAA TCCAACTTGGTTG-3′
yU3* [helix 1b′: P]	YU3BP	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGAGAGAAC AAAAGAGCCACTGAATCCAACTTGGTTG-3′
yU3* [helix 1b′: D]	YU3BD	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGACTCTTC AATTCTCCCACTGAATCCAACTTGGTTG-3′
yU3* [helix 1b′: PD]	YU3BPD	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGAGAGAAC AATTCTCCCACTGAATCCAACTTGGTTG-3′
yU3* [helix 1b′: LP]	YU3BLP	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGACTCTTG TTAAGAGCCACTGAATCCAACTTGGTTG-3′
yU3 [A70]	YU3A70	5′-CCAAACCTTTGGTTTTAAACAATTTAGAAAAGGAAAAAAGTGGTTGGATTCAGTGGCTCT TTTGAAGAGTCAAAG-3′
yU3 [ACD]	YU3ACD	5′-CCAAACCTTTGGTTTTAAACAATTTAGAAAAGGAAAAAAGTGGTTTCGCTCAGACATCTT TTGGAATCCATCATCATC-3′
yU3* [D6]	YU3D6	5′-TGACTCTGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGACAAAGA GCCACTGAATCCAACTTGGTTG-3′
yU3* [P6C]	YU3P6C	5′-TGACTCTGTCGACGTCGACGTACTTCATAGGATCATTTCTATAGGAATCGTCACTCTTTGA CCCCCCCTCTTCAAAAGAGCCACTGAATCCAACTTGGTTG-3′

with Milli-Q water, and resuspended in NET-2. Protease inhibitors (2 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.5 mM PMSF) were included in lysates. Cells were lysed by vigorous vortexing (5 × 45 s) with 0.45–0.5-mm glass beads. The lysate was cleared by centrifugation for 10 min at 13,000 × *g*. Immunoprecipitation was performed for 1 h at 4 °C using lysate prepared from the equivalent of 5 OD_{600} units of cells. RNA was recovered by extraction with PCA (phenol:chloroform:isoamyl alcohol; 25:24:1) and ethanol precipitated.

Northern blotting

Northern blotting was performed as described previously (Samarsky & Fournier, 1998). The recovered RNA was separated on 8% denaturing polyacrylamide gels, transferred to a Zeta-Probe membrane (Bio-Rad Laboratories), and analyzed by northern blotting with antisense oligonucleotide probes. Oligonucleotides used for probing U3 RNA included: SD13* (5′-CGGCTTAGGCTAAGCTAAGGCCAG-3′), which hybridizes to U3* and all its mutant derivatives but not to wild-type U3; and SD74 (5′-ATCAAGATCATCGCGCCGGTTTCTCAC TCTGGGGTAC-3′), which recognizes all U3 forms.

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