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The 5′ and 3′ domains of yeast U6 snRNA: Lsm proteins facilitate binding of Prp24 protein to the U6 telestem region

DANIEL E. RYAN, SCOTT W. STEVENS, and JOHN ABELSON
Division of Biology 147-75, California Institute of Technology, Pasadena, California 91125, USA

ABSTRACT

The 5′ and 3′ domains of yeast U6 snRNA contain sequences that are thought to be important for binding to Prp24 and Lsm proteins. By extensive mutational analysis of yeast U6 snRNA, we confirmed that the 3′ terminal uridine tract of U6 snRNA is important for U6 binding to Lsm proteins in yeast. Binding of Prp24 protein to U6 RNA is dependent on or is strongly enhanced by U6 binding of Lsm proteins. This supports a model for U6 snRNP assembly in which U6 RNA binds to the Lsm2–8 core prior to binding Prp24 protein. Using compensatory base-pairing analysis, we show that at least half of the recently identified U6 telestem as well as a nucleotide sequence in the other half of the telestem are important for binding of U6 RNA to Prp24 protein. Surprisingly, disruption of base pairing in the unconfirmed half of the telestem enhanced U6–Prp24 binding. Truncation of the entire 3′ terminal domain or nearly the entire 5′ terminal domain of yeast U6 allowed for detectable levels of splicing to proceed in vitro. In addition to gaining knowledge of the function of the 5′ and 3′ domains of yeast U6, our results help define the minimal set of requirements for yeast U6 RNA function in splicing. We present a revised secondary structural model of yeast U6 snRNA in free U6 snRNPs.

Keywords: Lsm4; pre-mRNA; snRNPs; spliceosome; splicing; U6 pseudoknot

INTRODUCTION

Splicing of pre-messenger RNA transcripts of eukaryotic genes is carried out in ribonucleoprotein particles called spliceosomes (reviewed in Nilsen, 1998; Burge et al., 1999; Hastings & Krainer, 2001). These are composed of the pre-mRNA substrate, the spliceosomal snRNAs (U1, U2, U4, U5, and U6 snRNAs), and about 70 known proteins, a number that is thought to be close to the actual total in a typical spliceosome (see Stevens et al., 2002). A primary driving force for research in the field has been the overarching goal of understanding the specific roles of the spliceosomal snRNAs and their associated proteins in catalyzing the pre-mRNA splicing reaction. The importance of U6 snRNA in the splicing reaction is supported by several lines of evidence, some of which are briefly outlined here. U6 snRNA is the most highly conserved snRNA component (Brow & Guthrie, 1988), and it is located at or near the active site of splicing catalysis during at least the first chemical step (Wassarman & Steitz, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993; Kim & Abelson, 1996; see also Valadkhan & Manley, 2001).

Sequences in the 5′ and 3′ domains of yeast U6 snRNA (nt 1–48 and 86–112) are thought to be important for binding to Prp24 and Lsm proteins (Fig. 1). Truncation of U6 RNA to remove nt 95–112 from its 3′ terminus hindered the normal binding of U6 RNA to Prp24 and Lsm proteins (Vidal et al., 1999). Only one mutation of yeast U6 RNA has been identified as having an effect on its binding to these proteins. The U6 point mutation, A91G, was found to inhibit the normal binding of Prp24 to U6 RNA in yeast extract (Shannon & Guthrie, 1991). We recently found that Prp24 and Lsm2–8 proteins are the only stably associated protein components of the yeast U6 snRNP (Stevens et al., 2001). Recombinant Prp24 protein binds to gel-purified yeast U6 transcript with a dissociation constant (K_d) of 100 nM, and the footprint of this protein bound to yeast U6 RNA has been mapped, primarily to the region of nt 30–65 (Ghetti et al., 1995). The normal binding of Prp24 protein to U6 is important for formation of U4-U6 di-snRNP during spliceosome assembly and recycling (Shannon & Guthrie, 1991; Jandrositz & Guthrie, 1995; Raghunathan & Guthrie, 1998a).

Reprint requests to: John Abelson, Division of Biology 147-75, California Institute of Technology, Pasadena, California 91125, USA; e-mail: abelsonj@caltech.edu.

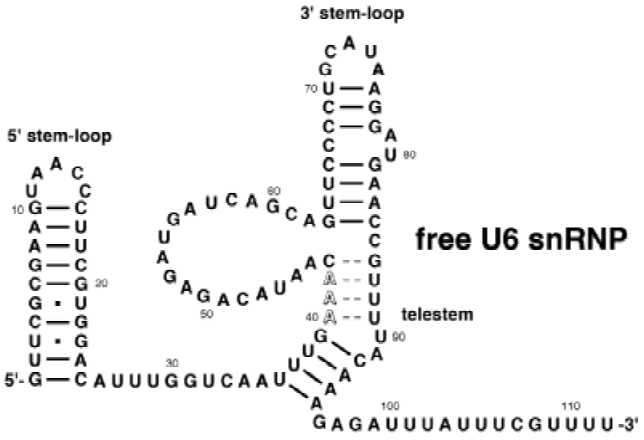

FIGURE 1. Secondary structure of U6 snRNA in yeast *S. cerevisiae*.

Mutations in the second and third RNA recognition motifs (i.e., RRM2 and RRM3) of Prp24 protein suppressed the cold-sensitive (cs) growth defect of a U6 point mutation that hyperstabilizes the 3′ stem-loop in U6 RNA, A62G (Vidaver et al., 1999), suggesting that these two RRMs of Prp24 may bind to U6 RNA and play a role in unwinding the 3′ stem-loop. Similarly, mutations in RRM3 of Prp24 suppressed the cs growth defect of a base-pair disrupting point mutation, U4.G14C, in stem II of U4-U6 di-snRNP (Shannon & Guthrie, 1991), suggesting that Prp24 may play a role in U4-U6 snRNA annealing. Both of these cs mutations in U6 and U4 snRNAs were suppressed by U6 point mutations that colocalized to two regions of U6 snRNA (Fortner et al., 1994). The sequences of these two regions of U6, that is, nt 36–43 and 86–95, are mutually complementary and could form an RNA duplex called the U6 telestem (Vidaver et al., 1999). Vidaver et al. found support for the existence of the U6 telestem in their genetic studies of U6 sequences in yeast. In the presence of the cs mutation U6 A62G, the suppressor (of cs) effect of a pair of U6 mutations that disrupt the U6 telestem was reverted by including a complementary pair of U6 mutations that can restore telestem base pairing. In additional experiments, the same disruption mutations of the U6 telestem were found to exacerbate the temperature-sensitive (ts) growth defect of mutant *prp24-R158S* (with a point mutation in RRM2 of Prp24). However, by including mutations that can restore telestem base pairing, this exacerbation effect was reversed. These experiments suggest that the U6 telestem exists in vivo and that it may interact with Prp24 protein. Immunoprecipitation of Prp24 from yeast extracts carrying the cs mutation in U4 snRNA, U4.G14C, showed an aberrantly high level of wild-type Prp24 protein bound to the mutant U4-U6 snRNP (Shannon & Guthrie, 1991). The combined results suggest that Prp24 binds to U6 RNA via its RRMs and promotes U4-U6 snRNA annealing for spliceosome assembly. A hypothetical mechanism is that the RRMs of Prp24, especially RRM2, may bind to the U6 telestem and subsequently release the telestem to allow initiation of U4-U6 snRNP assembly (Vidaver et al., 1999). This release could destabilize base pairing in the U6 3′ stem-loop duplex that must unwind and base pair to U4 snRNA to generate U4-U6 snRNP for assembly into spliceosomes.

Prp24 has been shown to interact via two-hybrid assays with Lsm2, Lsm5, Lsm6, and Lsm7 proteins (Fromont-Racine et al., 2000). The Lsm ("like Sm") proteins belong to a family that includes the evolutionarily and functionally related Sm proteins. The Sm proteins form a doughnut-shaped heteroheptamer that binds to each of the spliceosomal snRNAs except U6 snRNA (for Sm core structures, see Kambach et al., 1999; Collins et al., 2001; Mura et al., 2001; Törö et al., 2001). U6 snRNA is associated with Lsm2–8 proteins, and these are thought to form a doughnut-shaped heteroheptamer bound to U6 (Achsel et al., 1999; Mayes et al., 1999; Vidal et al., 1999; Bouveret et al., 2000). U6 binding to the Lsm core appears to stabilize U6 snRNA against degradation (Mayes et al., 1999). Lsm proteins are sufficient to promote the annealing of human U4 and U6 RNA transcripts in vitro, suggesting that these proteins may play a similar role in the annealing of U4 and U6 snRNPs in vivo (Achsel et al., 1999). For human U6 snRNA, the 3′ terminal 12 nt were found to be necessary and sufficient for binding to the Lsm proteins, and the tract of five uridines at the 3′ end was shown to be an important determinant for this binding (Achsel et al., 1999). In contrast, for yeast U6 snRNA, the last 18 nt at the 3′ terminus are necessary but not sufficient for U6 binding of the Lsm core (Vidal et al., 1999). Vidal et al. found that the 3′ terminal domain of yeast U6 RNA is necessary not only for binding to Lsm proteins but also for U6 crosslinking to Prp24 and other U6-proximal splicing proteins. The 3′ terminal domain of yeast U6 (nt 86–112) is mostly divergent, although it maintains the ability to form U2-U6 helix II and has a short uridine tract at its 3′ end, as found in other organisms.

In this report of mutations in yeast U6 snRNA that affect splicing (see also Ryan & Abelson, 2002), we introduced mutations or deletions in the 5′ and/or 3′ domains of yeast U6 RNA, especially in the 5′ and 3′ telestem sequences, in the U6 sequence of U2-U6 helix II and in the uridine tract at the 3′ end of U6. We found that specific sequences in the 5′ and 3′ domains of yeast U6 snRNA are important for binding to Prp24 and/or Lsm proteins. However, despite their importance for Prp24 and Lsm binding, most of these sequences were not found to be important for splicing in vitro. It has been known that yeast U6 lacking nt 95–112 from its 3′ end is able to reconstitute 34% of full-length U6 RNA splicing activity (Fabrizio et al., 1989), despite the loss of one telestem base pair plus 8 of 11

base pairs of U2-U6 helix II and the entire 3′ terminal uridine tract. We have analyzed these specific sequences individually for their effects on assembly of U6-containing snRNPs, on spliceosome assembly, and on splicing in vitro. To further our knowledge of the chemical requirements for U6 function in splicing, we also defined the largest truncations of the 5′ and 3′ terminal domains of yeast U6 RNA that allow for detection of splicing in vitro.

RESULTS

Truncation of the 5′ and 3′ terminal domains of yeast U6 snRNA

The splicing activities of a few 3′ truncated yeast U6 RNAs have been previously reported. A U6 transcript lacking 18 nt from the 3′ end, U6.1–94 (truncated at the *Fok*I restriction endonuclease site), was found to reconstitute 34% of full-length U6 splicing activity when added to U6-depleted yeast extract, whereas U6.1–73 showed no splicing activity in the in vitro assay (Fabrizio et al., 1989). Studies in *Saccharomyces cerevisiae* of a 3′ truncated U6 gene, U6.1–90, on a centromere plasmid as the sole copy of the U6 gene were not viable (Bordonné & Guthrie, 1992). We decided to test further truncations of the active U6.1–94 RNA using the same in vitro splicing assay as Fabrizio et al. (1989).

All U6 RNAs discussed in this report were prepared synthetically by template-directed ligation of synthetic RNA oligonucleotides using T4 DNA ligase. To test the splicing activity of full-length and 3′-truncated U6 RNAs, splicing assays were conducted in yeast extracts using [α-^{32}P]uridine-labeled actin pre-mRNA as the splicing substrate. In the assay, endogenous U6 snRNA in the extract is depleted by oligo-directed RNase H digestion using a deoxynucleotide (d1) complementary to nt 28–54 in yeast U6 RNA (Fabrizio et al., 1989). Synthetic U6 RNAs are then added to reconstitute U6 snRNPs in vitro, and these RNAs are also radiolabeled with ^{32}P to monitor their stability in the yeast extract.

We prepared the U6.1–94 truncated RNA studied previously as well as further truncations, U6.1–91, 1–88, 1–86, 1–84, 1–81, and 1–80. In preliminary experiments, U6.1–94, 1–91, and 1–88 reconstituted 35–40% of full-length U6 splicing activity (Table 1, part A; data not shown) as reported previously for U6.1–94 (Fabrizio et al., 1989), whereas the slightly shorter construct U6.1–86 reconstituted only 20% of the splicing activity of full-length U6 (shorter U6 RNAs were tested subsequently; see below).

We observed that the full-length U6 RNAs were routinely degraded in yeast extracts to shorter RNAs equivalent in length to truncations at or near the 3′ end of U2-U6 helix II. Truncated RNAs U6.1–94, 1–91, 1–81, and 1–80 showed partial or extensive degradation by the loss of one to a few nucleotides (e.g., U6.1–94 was degraded completely to U6.1–91/90) whereas U6.1–88, 1–86, and 1–84 were extensively degraded to lengths matching the gel mobility of U6.1–81 (data not shown), suggesting that this latter group of U6 RNAs may be degraded at their 3′ ends until double-stranded U4-U6 stem II impedes further exonucleolytic degradation (U4-U6 stem II base pairing includes U6 nt 80 at the end of the duplex). To block single-strand specific, exonucleolytic degradation of synthetic U6 RNAs in extract, we incorporated four 5′-3′ phosphorothioate linkages between the last 5 nt at the 3′ end of full-length U6 (U6.thioP) and two such phosphorothioate linkages at the 3′ end of U6.1–85 (U6.1–85.thioP) via chemical synthesis (thus each phosphorothioate is racemic). Because previous work had shown that an (R_P)-phosphorothioate incorporated at the 79–80 linkage in U6 blocked splicing completely (Fabrizio & Abelson, 1992), we decided to use the U6.1–81 and 1–80 RNAs without 3′ end phosphorothioates, as these were significantly less degraded in extract than the slightly longer U6 RNAs, as mentioned above. The 3′ terminal phosphorothioate linkages substantially blocked extract-dependent degradation of exogenously added U6 RNAs (Figs. 2 and 3; data not shown), presumably by inhib-

TABLE 1. Splicing yields for truncated U6 RNAs in yeast extract.

Truncated U6 RNAs	Relative splicing yields[a]	Mole ratio of lariat:mRNA
Part A		
U6.1–86	20%[b]	1:1.2
U6.1–88	34%[b]	1:1.1
U6.1–91	41%[b]	1.2:1
U6.1–94	36%[b]	1:1.1
U6 wild-type (1–112)	100%[b]	1:3.3
Endogenous U6[c]	250%[b]	1:4.1
Part B		
U6.1–80	0%[d]	n/a
U6.1–81	0%[d]	n/a
U6.1–85.thioP[e]	4%[d]	1.0-1.3:1[d]
U6 wild-type[f]	100%	1:2.6[d]
Endogenous U6[c]	180%	1:6.0
Part C		
U6.39-112[f,g]	23%, 31%[d]	1.4–1.6:1[d]
U6 wild-type[f]	100%	2.1–2.4:1[d]

[a] Splicing yields for reconstituted, synthetic U6 RNAs and endogenous wild-type U6 RNA are normalized relative to the yield for reconstituted, synthetic full-length U6 RNA (U6 wild-type) as a control.
[b] In these particular experiments, splicing yields are based solely on mRNA products. Normally, a splicing yield is based on first- and second-step splicing products. However, the U6 truncations showed discrepancies in lariat degradation.
[c] Endogenous wild-type U6 snRNA in yeast extract that was not U6-depleted or reconstituted with synthetic U6 RNA.
[d] For duplicate samples.
[e] The last two 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.
[f] The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.
[g] The first seven 5′-3′ linkages at the 5′ terminus are racemic phosphorothioates.
n/a: not applicable.

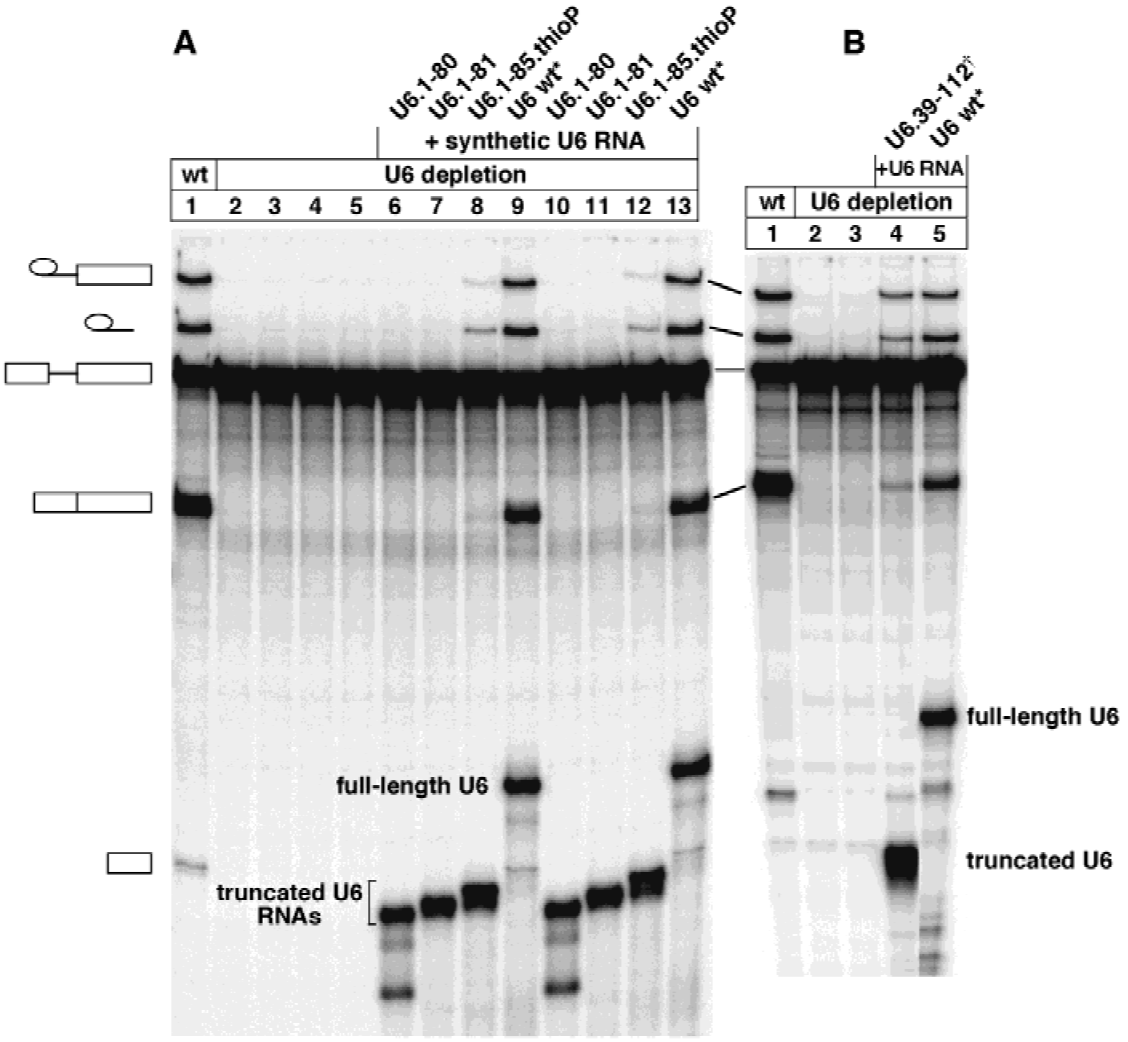

FIGURE 2. Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with truncated and full-length U6 RNAs. Yeast splicing extract was treated (**A**: lanes 2–13; **B**: lanes 2–5) or not treated (lane 1) with a DNA oligonucleotide (d1) complementary to U6 snRNA. After incubation at 34 °C to allow native RNase H digestion of endogenous U6 snRNA, aliquots of the U6-depleted extract were reconstituted by adding synthetic, ^{32}P-labeled U6 RNA as indicated. †In synthetic U6.39-112 RNA, the first seven 5′-3′ linkages at the 5′ end and the last four such linkages at the 3′ end were racemic phosphorothioates to block degradation via endogenous exonucleases. Synthetic U6 RNA labeled with an ∗ contained four such phosphorothioate linkages at its 3′ end; U6 RNA labeled as .thioP had two such linkages at its 3′ end. Splicing of ^{32}P-labeled actin pre-mRNA substrate was assayed at 25 °C for 30 min. This substrate has one intron and gives rise to exon1 and lariat-exon2 intermediates after the first chemical step of splicing and to excised lariat intron and spliced mRNA products after the second chemical step (as indicated pictorially). Total nucleic acid for each sample was separated on a denaturing polyacrylamide gel. In **A**, U6-depleted extract was assayed twice in lanes 2 and 3 for residual splicing activity, and the averaged background signal was used to correct the levels of splicing products assayed in lanes 6–9. A replicate U6-depleted extract was assayed in lanes 4 and 5 and was used for lanes 10–13. Thus, the assays for lanes 6–9 were repeated in lanes 10–13. In **B**, samples were prepared as in **A**. As for all of our presentations of individual gels, even those which were obviously cut and pieced together as in **B**, all of the samples for the gel were prepared concurrently and were separated on one single gel.

iting single-strand specific exonucleases in the extracts (Pandolfi et al., 1999). We tested a variety of U6 mutants with and without phosphorothioate linkages at their 3′ termini, and we found that the phosphorothioate modifications had no detectable effect on splicing activity in vitro (see below). Therefore, at least for full-length U6 RNAs, the splicing activity of U6 with 3′ terminal phosphorothioates reflects equivalently the splicing activity of nonphosphorothioylated U6 RNA.

In experiments using the phosphorothioate-substituted U6 RNAs to block 3′ end degradation, we assayed the splicing activity of 3′-end-truncated yeast U6 RNAs (Fig. 2A, Table 1, part B). We observed that full-length synthetic U6.thioP reconstituted 55% of wild type splicing activity in duplicate samples (Fig. 2A, cf. lane 1 and lanes 9 and 13) as is typical (Fabrizio et al., 1989; our experiments). The truncation U6.1–85.thioP had the largest 3′-end truncation that reconstituted detectable splic-

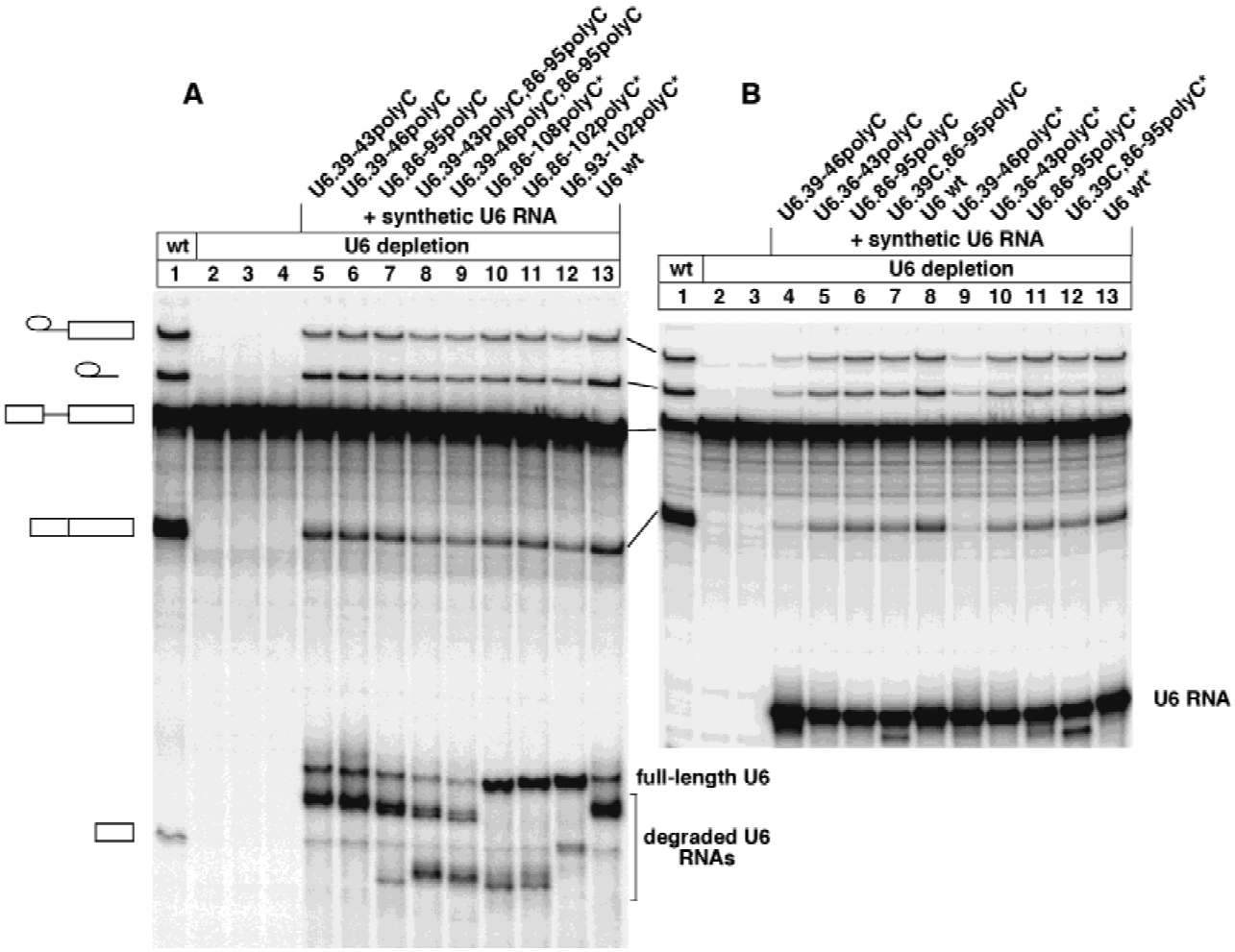

FIGURE 3. Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95) and in the 3′ terminal domain (nt 86–112) of yeast U6 RNA. Samples were prepared as in Figure 2. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates. In **A**, three aliquots of the U6-depleted extract were assayed and used for background correction as in Figure 2. The assays in **A** were performed three years before those in **B** using the same stock of extract stored at −80 °C.

ing activity, and it produced a 4% splicing yield in duplicate samples (Fig. 2A, lanes 8 and 12). In contrast, further truncations of the U6 RNA, that is, U6.1–81 and 1–80, effectively blocked splicing activity as no splicing products were detected (Fig. 2A, lanes 6, 7, 10, and 11).

In the original study of the splicing activity of U6.1–94 in vitro, it was noted that the ratio of lariat intron versus spliced mRNA was 3–3.8 times higher than for most U6 RNAs (Fabrizio et al., 1989). We were able to reproduce this result exactly, and essentially the same results were obtained for U6.1–91, 1–88, 1–86 and 1–85.thioP (data not shown). For each of these truncated U6 RNAs, quantitation of the splicing products (on a gel exposed to a PhosphorImager screen) including the unusually abundant lariat intron, showed that the mole ratio of lariat intron versus mRNA was very close to 1:1 in each case (ranging from 1.3:1 to 1:1.2; Table 1). In contrast, the mole ratio of lariat intron versus mRNA for full-length U6 reconstitutions was generally close to 1:3 in these experiments (ranging from 1:2.6 to 1:3.3). As the lariat intron and spliced message must be nascently generated in equimolar amounts, the lariat is typically degraded about threefold more than the spliced message in our wild-type extracts reconstituted with full-length U6. Surprisingly, the truncated U6 RNAs apparently blocked the normal degradation of lariat intron.

In previous work to define the minimal sequence requirements for U6 function, human U6 snRNA was successively truncated at its 5′ end, and it was found that deletion of 23 nt from the 5′ terminus had no deleterious effects on splicing in HeLa extract (Wolff & Bindereif, 1992). Up to 37 nt were deleted from the 5′ terminus of human U6 RNA, and it still maintained detectable splicing activity. However, this maximal truncation

showed only a <10% yield of splicing products. A similarly large truncation had not been tested in yeast U6. Therefore, we constructed a synthetic U6 RNA lacking the first 38 nt of the 5′ end of yeast U6 and containing seven 5′-3′ phosphorothioate linkages between the first 8 nt at the truncated 5′ end and four such linkages at the 3′ end to block exonucleolytic degradation in yeast extracts. The U6.39–112 truncated RNA reconstituted 23–31% of full-length U6 splicing activity (Fig. 2B, lane 4; Table 1, part C). Therefore, as reported for human U6 RNA, yeast U6 does not require the 5′ terminal domain for detectable splicing activity.

Extensive mutation of the 3′ domain and telestem sequences of yeast U6 snRNA

We have shown that truncation of the entire U2-U6 helix II region and 3′ tail of U6 in U6.1–91 reconstituted about 30% of full-length U6 splicing activity and inhibited the normal degradation of intron lariat. Truncation of the entire 3′ domain (nt 86–112), which removes the entire 3′ sequence of the putative U6 telestem (nt 86–95), reduced splicing activity to 4% of full-length U6. To assess the loss of sequence information separately from any effects of shortening the U6 RNA, we prepared U6 RNAs with multiple cytidine mutations in the 3′ terminal domain, as there are only two cytidines among the 27 nt in the 3′ domain of wild-type yeast U6. In preliminary studies, we found that polycytidine (polyC) mutations in the U2-U6 helix II region of U6 led to extensive degradation of these RNAs in yeast extract, as we had observed for truncated U6 RNAs (see above). By synthetically incorporating four α-phosphorothioate nucleotides at the 3′ termini of the U6 RNAs with helix II mutations, degradation by single-strand specific, exonuclease activity in the extract was almost completely blocked (theoretically 94% blocked based on racemic phosphorothioates).

The polycytidine mutations studied in our experiments included a complete polycytidine substitution of the U6 sequence of U2-U6 helix II in mutant U6.93–102polyC.thioP (.thioP means that 5′-3′ phosphorothioates were incorporated at the 3′ end). In our initial studies, the telestem mutations were U6.39–43polyC, U6.39–46polyC and U6.86–95polyC and the two combinations of these upstream and downstream telestem mutations. (Nucleotides 36–38 of the telestem sequence were contained on another piece of U6 in our original ligation scheme and were not mutated in the initial studies.) The combined telestem and helix II mutations were U6.86–102polyC.thioP and U6.86–108polyC.thioP. The 86–108polyC mutation is a complete conversion of the entire 3′ terminal domain to polycytidine, except for maintenance of the four 3′ terminal uridines. As mentioned, the terminal uridine tract is important for binding of U6 RNA to Lsm proteins in human cells, and it was therefore implicated for U6 binding to Lsm proteins in yeast. We investigated mutations of the 3′ terminal uridine tract separately (see below).

The effects of these polyC mutations of helix II, the telestem, and the 3′ terminal domain on splicing in vitro are presented in Figure 3A and Table 2, Column A. Extensive mutation of the 5′ sequence of the telestem to polycytidine or complete mutation of the 3′ telestem sequence to polycytidine resulted in a splicing yield that was 65–85% that of wild-type U6 (Fig. 3A, lanes 5–7). Nearly complete mutation of the entire telestem (for U6.39–43polyC,86–95polyC) or of the entire 3′ terminal domain (for U6.86–108polyC.thioP, etc.) resulted in a relative splicing yield of 35–55% (Fig. 3A, lanes 8–12). The effects of mutating both the upstream and downstream sequences of the telestem were somewhat more deleterious than expected from the effects of each sequence mutated separately. In Figure 3A, mutating the two sequences of the telestem simultaneously was about 1.5 times more deleterious than expected from the separate mutations.

To investigate complete disruption of the U6 telestem, we mutated the entire upstream or downstream telestem sequence to polycytidine in the mutants U6.36–43polyC and U6.86–95polyC. To disrupt every G-C base pair in the telestem, we also tested these two polyC mutations in combination with a cytidine mutation of the only remaining guanine within the telestem sequences. These two mutants, U6.36–43polyC,86C and U6.39C,86–95polyC, have no possibility of a Watson–Crick base pairing within the telestem sequences. In our preliminary experiments, when the U6 reconstitutions were successful but not optimized, it was clear that there was no discernible difference between U6.36–43polyC and U6.36–43polyC,86C in replicate splicing assays; however, there was a small but reproducible difference in splicing activity between the U6.86–95polyC and U6.39C,86–95polyC mutants. The results for these telestem disruption mutations are presented in Figure 3B and Table 2, Column B. The combined results (Table 2, Columns A and B) show that complete disruption of the telestem resulted in a 1.5–2.5-fold reduction in splicing activity (Fig. 3B, lanes 5–7, 10–12). We tested the mutations in Table 2, Column B with and without phosphorothioate linkages at their 3′ termini and found that these modifications had no detectable effect on splicing activity. For comparison to our initial data (Table 2, Column A), we also assayed the in vitro splicing activity of U6.39–46polyC. Splicing activity for this particular polyC mutant, regardless of whether stabilized against degradation in extract, was the most variable, ranging from 13 to 85% in our assays. Similarly, our polycytidine mutations that overlap the U6.39–46polyC region, that is, the U6.36–43polyC and U6.36–43polyC,86C mutations, produced a relatively broad range of splicing yields (14–48%, including data not

TABLE 2. Splicing yields for mutations in the 3′ terminal domain and telestem sequences of U6 RNA in yeast extract.

Mutated U6 RNAs	Relative splicing yields[a]		Growth in yeast[c]
	Column A	Column B[b]	
U6.39–43polyC	88%, 55%[d]	—	n.t.
U6.39–46polyC	85%, 60%[e], 42%[d]	16%, 13%[f]	lethal
U6.36–43polyC	—	48%, 45%[f]	+++
U6.86–95polyC	68%, 41%[e], 64%[d]	67%, 69%[f]	+++
U6.39C,86–95polyC	—	53%, 52%[f]	n.t.
U6.39–43polyC,86–95polyC	42%	—	n.t.
U6.39–46polyC,86–95polyC	35%, 35%[e]	—	n.t.
U6.36–43polyC,86–95polyC	—	—	lethal
U6.86–108polyC[g]	50%, 52%[e]	—	+/−
U6.86–102polyC[g]	55%	—	+/−
U6.93–102polyC[g]	36%, 43%[e]	—	+++
U6 wild type	100%	100%	+++

[a]Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.
[b]The experiment for column B is shown in Figure 3B.
[c]Growth was assayed at 16, 23, 30 and 37 °C; +++: wild-type growth at all temperatures, +/−: a subpopulation of slow growing colonies appeared reproducibly at all temperatures except 37 °C, lethal: no growth at all temperatures, n.t.: not tested.
[d]Yield in a replicate experiment using the same extract three years later.
[e]Yield in a duplicate experiment.
[f]For the second yield listed, the last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.
[g]The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.

shown). The range of splicing yields for freshly ligated U6.86–95polyC (Table 2, Columns A and B) is more typical of the range we observe in replicate trials. The unusually wide range observed for U6.39–46polyC may be due to variable misfolding of the mutant RNA. We note that in the more recent experiments (Table 2, Column B), the splicing yields determined for U6.39–46polyC and the other U6 telestem disruption mutations appear to correlate best with the growth phenotypes we observed for these mutations in yeast cells (Table 2; see below).

To determine whether these U6 telestem and 3′ domain mutations are viable in yeast, we transformed a haploid strain, HM1 (Madhani et al., 1990), containing a chromosomal deletion of the U6 gene (*SNR6*) that also contains a plasmid copy of the U6 gene plus counterselectable URA3 marker gene. Mutant U6 plasmids were constructed as described in Materials and Methods and transformed into yeast strain HM1, and the wild-type U6 gene was eliminated by selective growth on media containing 5-fluoroorotic acid (5-FOA; Boeke et al., 1987). The U6 snRNA mutations tested in vivo and the resulting growth phenotypes are presented in Table 2. Upon counterselection of the wild-type U6 gene in strains containing U6.86–108polyC or U6.86–102polyC plasmids, we observed a substantially reduced colony number with respect to wild-type controls and other viable U6 mutations, and the colonies that did arise grew much more slowly. To determine whether these colonies resulted from revertant or compensatory mutations in the mutant U6 genes, plasmids harvested from six colonies of each of the two slow-growing strains were transformed into *Escherichia coli*, and the recovered U6 genes were sequenced for the 12 plasmids. We found no revertant or compensatory mutations within the U6.86–108polyC and U6.86–102polyC genes. Therefore, the slow-growing colonies are viable (1) perhaps without any additional mutations or changes in transcription levels, (2) as a result of spontaneous, extragenic suppressor mutations that were not identified, or (3) as a result of changes in the level of the mutant U6 RNA produced. Regardless, our results demonstrate that neither the U6 sequence of U2-U6 helix II nor the U6 telestem is required for splicing activity or for growth at various temperatures in yeast. For most studies of snRNA mutations, the mutational effects found in yeast cells have correlated well with the effects observed in yeast extracts and vice versa (Fabrizio & Abelson, 1990; Madhani et al., 1990; Madhani & Guthrie, 1992, 1994; McPheeters & Abelson, 1992; McPheeters, 1996). The viability of U6 telestem mutations in yeast correlates well with the modest effects of the telestem disruption mutations on pre-mRNA splicing in vitro (Table 2).

Both yeast and mammalian U6 snRNAs have a short uridine tract at their 3′ ends that is thought to be an essential determinant for binding to Lsm proteins (Achsel et al., 1999; Vidal et al., 1999). We designed an experiment to test the splicing effects of mutating the four terminal uridines in yeast U6 RNA. In two mutants,

these four uridines were entirely mutated to adenines or cytidines. Conversely, to eliminate all other sequence information in the 3′ terminal domain, we also tested our U6 mutant that possesses polycytidine mutation of the entire 3′ domain except for the four terminal uridines. In two additional variations, the terminal uridines were also mutated, either to polyadenine or polycytidine. To stabilize the 3′ terminus against exonucleolytic degradation, we incorporated four 5′-3′ phosphorothioate linkages between the last 5 nt at the 3′ end of each of these U6 RNAs. Splicing assays for the 3′ terminal mutants are presented in Figures 4A and 5A and Table 3. Mutation of the entire 3′ terminal domain except for the last four uridines (i.e., nt 86–108) resulted in two- to threefold less splicing activity than for wild-type U6 (Fig. 3A, lane 10; Fig. 4A, lane 14; Table 2, part A; Table 3), but further mutation to include the entire 3′ terminal uridine tract caused splicing activity to drop to barely detectable levels (2% yields, Table 3; Fig. 4A, lanes 12 and 13). Surprisingly, mutation of only the 3′ terminal uridine tract to polyadenine or polycytidine had a rather minor effect on splicing in vitro (Fig. 5A, lanes 3 and 4; Table 3).

To ensure that a polyuridine tract was not added to the 3′ ends of the U6 uridine tract mutants by some activity in the extract, we conducted a simple experiment to check whether the U6 uridine tract mutants could be uridinylated in extract. Our standard depletion of endogenous U6 snRNA in 10 μL of epitope-tagged Prp24$(HA)_3$ extract in splicing buffer was followed by addition of 75 μCi of [α-^{32}P]UTP and then addition of 25 fmol of wild-type U6 or 3′ uridine tract mutant U6 RNA (U6.109–112polyA/polyC). After incubation at 23 °C for 20 min, reconstituted U6 snRNPs in these 25-μL

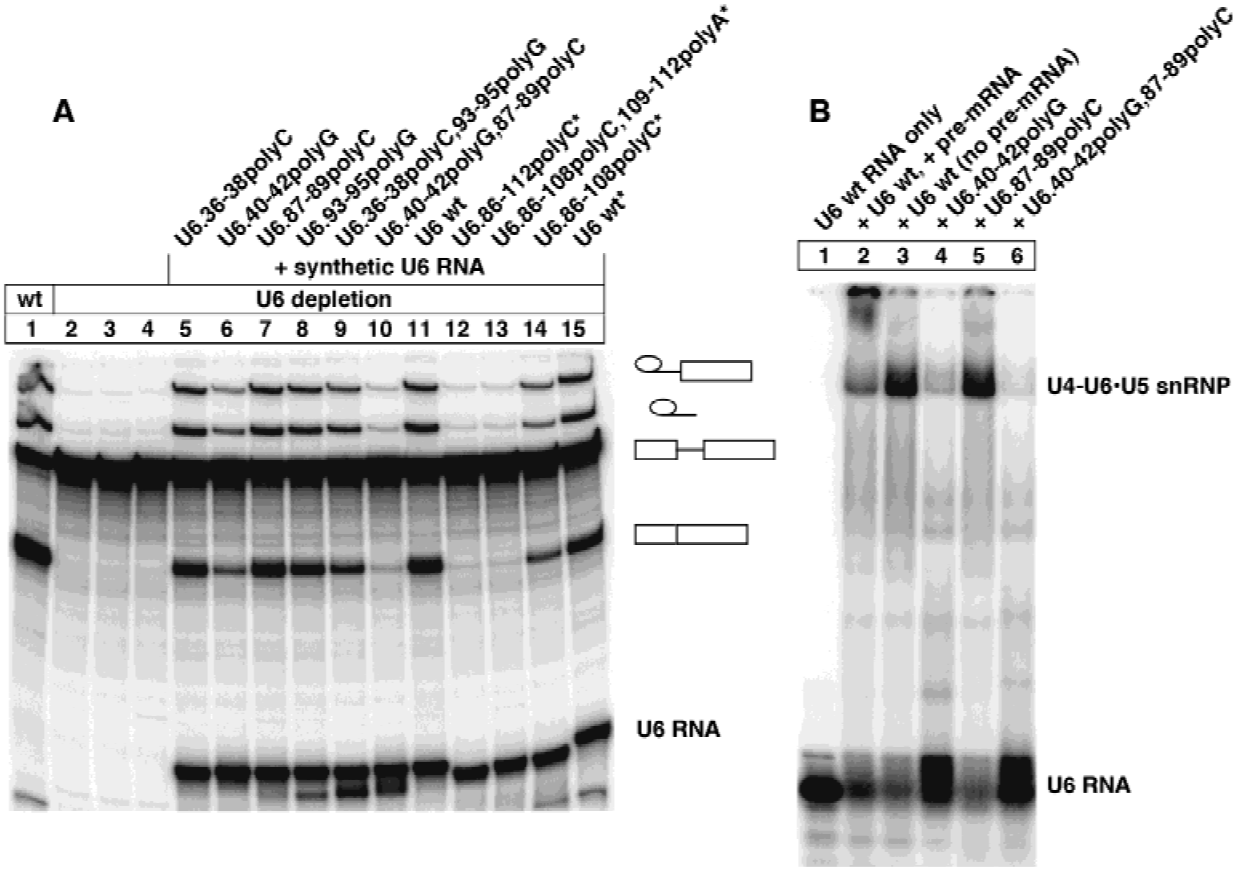

FIGURE 4. **A**: Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95) of yeast U6 RNA, including potentially hyperstabilizing mutations (lanes 9 and 10). Mutations were also introduced in the yeast U6 3′ terminal domain (nt 86–112) and terminal uridine tract (nt 109–112). Samples were prepared as in Figure 2. Three aliquots of the U6-depleted extract were assayed and used for background correction as in Figure 2. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates. **B**: U4-U6•U5 snRNP assembly for U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95). Yeast splicing extract was treated with d1 oligonucleotide to digest endogenous U6 snRNA as described in Figure 2. Aliquots of the U6-depleted extract were reconstituted by addition of 2 fmol of synthetic, ^{32}P-labeled U6 RNA as indicated. For lanes 3–6, samples were incubated at 23 °C for 20 min and then loaded onto a nondenaturing 4% polyacrylamide (79:1) gel for separation of the U6 snRNP-containing complexes as well as free U6 RNAs. For lane 2, actin pre-mRNA was added just prior to the 20-min incubation.

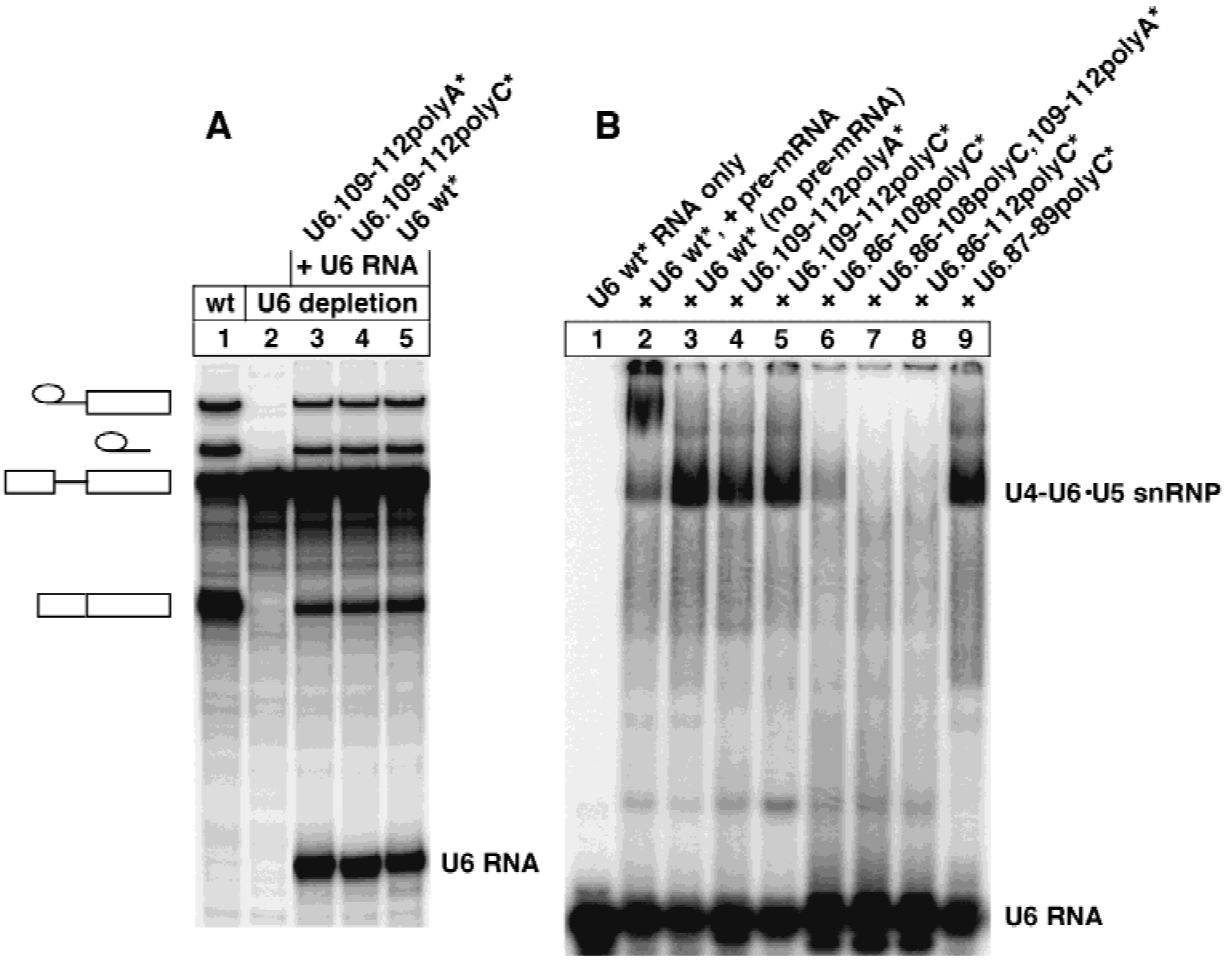

FIGURE 5. **A**: Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the 3′ terminal uridine tract (nt 109–112) of yeast U6 RNA. Samples were prepared as in Figure 2. All samples were prepared concurrently and were separated on the same gel. **B**: U4-U6•U5 snRNP assembly for U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the 3′ terminal uridine tract (nt 109–112) and the 3′ terminal domain (nt 86–112) of yeast U6 RNA. Samples were prepared as in Figure 4B. In synthetic U6 RNAs marked with an $*$, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

samples were immunoprecipitated with 0.5 μg of 12CA5 antibody on protein A-Sepharose beads (a background control lacked antibody). Scintillation counting of the washed beads showed that neither wild-type U6 nor the 3′ uridine tract mutants were uridinylated by [α-^{32}P]UTP under splicing conditions (data not shown). Therefore, no alternative 3′ terminal uridine tract was added to our mutant U6 RNAs by factors in the yeast extract.

Depletion of Prp24 protein in yeast extract affects the assembly of U6 RNA into U4-U6•U5 tri-snRNPs

Raghunathan and Guthrie (1998a) have shown that Prp24 catalyzes the reassembly of U4-U6 snRNP during spliceosome recycling for successive rounds of splicing in yeast extracts. They could not readily determine whether Prp24 is important for the biogenesis of U6-containing snRNPs, as these complexes were already present in their Prp24-depleted extract. To determine whether Prp24 is important for the production of U6-containing snRNPs in our extracts, we employed our U6 reconstitution method to assay for assembly of ^{32}P-labeled U6 RNA into U6 snRNP complexes. The standard protocol for RNase H depletion of endogenous U6 RNA is very effective at digesting the total endogenous U6 RNA in yeast extracts, including that in U4-U6-containing snRNPs (Fabrizio et al., 1989). We prepared extracts of a yeast strain (PRY112) carrying an epitope-tagged Prp24 gene as the sole copy of this essential gene. This strain was originally prepared and used by P. Raghunathan, and we received an aliquot of her extract for comparison with ours. For all samples in our experiments, we first depleted the endogenous U6 RNA using our standard protocol. Split samples were then either immunodepleted or mock depleted of epitope-tagged Prp24$(HA)_3$ protein using commercial

TABLE 3. Data summary for mutations in the 3′ terminal domain, 3′ stem-loop and telestem sequences of yeast U6 RNA.

Mutated U6 RNAs[a]	Splicing yields[b]	U4/U6–U5 yield[c]	Polyoma-Lsm4 I.P.[d]	Prp24(HA)$_3$ I.P.[e]
U6.36–43CpolyC,86C	28%[f] (14–45%)	18%[f,g] (15–23%)	39%, 33%[h]	2%, 2%[h]
U6.39C,86–95polyC	44%[f] (35–53%)	62%[f,g] (50–76%)	63%, 56%[h]	4%, 3%[h]
U6.86–112polyC	2%[i]	7%[j]	2%	8%
U6.86–108polyC,109–112polyA	2%[i]	6%[j]	2%	6%
U6.86–108polyC	34%[i] (34–52%)	20%[j]	24%	8%
U6.87–89polyC	102%, 114%[h]	105%[f] (103–109%)	42%	140%[f] (83–228%)
U6.109–112polyA	80%, 83%[h]	64%[k]	10%	7%, 9%[h]
U6.109–112polyC	77%, 81%[h]	76%[k]	9%	15%, 9%[h]
U6.U80G	5%	23%, 23%[h,l]	280%	940%, 900%[h]
U6 wild type	100%	100%	100%	100%

[a]The last four 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates, except for U6.U80G, which has no phosphorothioates.
[b]Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.
[c]Yield of U4/U6-U5 tri-snRNP assembled from reconstituted U6 RNA, separated by native gel electrophoresis and normalized relative to the U6 wild-type control.
[d]Relative amount of reconstituted U6 RNA coimmunoprecipitated on anti-polyoma protein G-Sepharose beads, normalized relative to the U6 wild-type control.
[e]Relative amount of reconstituted U6 RNA coimmunoprecipitated with 12CA5 antibodies on protein A-Sepharose beads, normalized relative to the U6 wild-type control.
[f]Average of four or more trials (range of yields).
[g]U4/U6-U5 tri-snRNP assembly gel showed aberrantly high levels of naked mutant U6 RNA not incorporated into U6 snRNPs and a minor accumulation of free U6 snRNP relative to the U6 wild-type control.
[h]Yield in a duplicate experiment.
[i]Yield from experiment in Figure 4A (range of yields including additional trials.
[j]U4/U6-U5 tri-snRNP assembly gel showed aberrantly high levels of naked mutant U6 RNA not incorporated into U6 snRNPs.
[k]U4/U6-U5 tri-snRNP assembly gel showed a minor accumulation of naked mutant U6 RNA relative to the U6 wild-type control.
[l]U4/U6-U5 tri-snRNP assembly gel showed aberrantly high levels of free U6 snRNP relative to the U6 wild-type control.

12CA5 antibodies (as in Raghunathan & Guthrie, 1998a). Western blotting showed that the epitope-tagged Prp24(HA)$_3$ protein was reproducibly immunodepleted in three trials to give postdepletion levels of Prp24(HA)$_3$ protein that were 15–25% of mock- and pre-depletion levels (data not shown). After depletion or mock depletion of Prp24 protein, all samples were treated with [α-^{32}P]-body-labeled wild-type U6 RNA to visualize the newly generated U6 snRNP complexes. Some U6-reconstituted samples were further treated with recombinant Prp24 protein. The ^{32}P-labeled U6 snRNP-containing complexes were separated on a native gel (Raghunathan & Guthrie, 1998a), and newly formed U4-U6•U5 tri-snRNP complex was clearly discernable as the slowest migrating and most intense complex on the gel and the only complex shifted (to slower mobility) by the addition of cold actin pre-mRNA (Fig. 6A), as observed by Raghunathan and Guthrie (1998a), although they used northern probes rather than ^{32}P-labeled U6 to visualize U6 snRNP complexes. In our experiments, quantitation of de novo tri-snRNP complexes revealed that the assembly of ^{32}P-labeled U6 RNA into U4-U6•U5 tri-snRNP complexes was catalyzed by Prp24 protein. For extracts depleted of both endogenous U6 RNA and Prp24 protein in three trials, addition of ^{32}P-labeled U6 RNA but not Prp24 protein produced only 14–16% as much de novo U4-U6•U5 tri-snRNP as produced in samples that were mock depleted of Prp24 but otherwise treated equivalently (Fig. 6A, cf. lanes 3 and 5). In split samples of the U6-, Prp24-depleted extracts, addition of both ^{32}P-labeled U6 RNA and recombinant Prp24 protein restored assembly of new U4-U6•U5 tri-snRNPs to levels that were 75–97% as high as for samples that were mock depleted of Prp24 but otherwise treated equivalently in three trials (Fig. 6A, cf. lanes 4 and 6). We conclude that Prp24 protein is important or required for catalyzing the assembly of naked U6 snRNA into U4-U6•U5 tri-snRNP in our extracts, presumably by binding to U6 snRNA to facilitate its incorporation into U4-U6 di-snRNP as demonstrated previously in vitro (Raghunathan & Guthrie, 1998a).

Mutations in the telestem sequences or in the 3′ terminal uridine tract of yeast U6 snRNA affect U6 RNA–protein binding and U4-U6•U5 tri-snRNP assembly

Having verified that Prp24 protein catalyzes the assembly of naked U6 RNA into U4-U6•U5 tri-snRNPs in vitro, we were interested in determining whether our U6 telestem and 3′ terminal domain mutations affect U6 binding of Prp24 protein and/or Lsm proteins and also whether tri-snRNP assembly is affected. Mass spectrometry analysis of purified yeast U6 snRNP indicated that free U6 snRNP has a relatively small complement

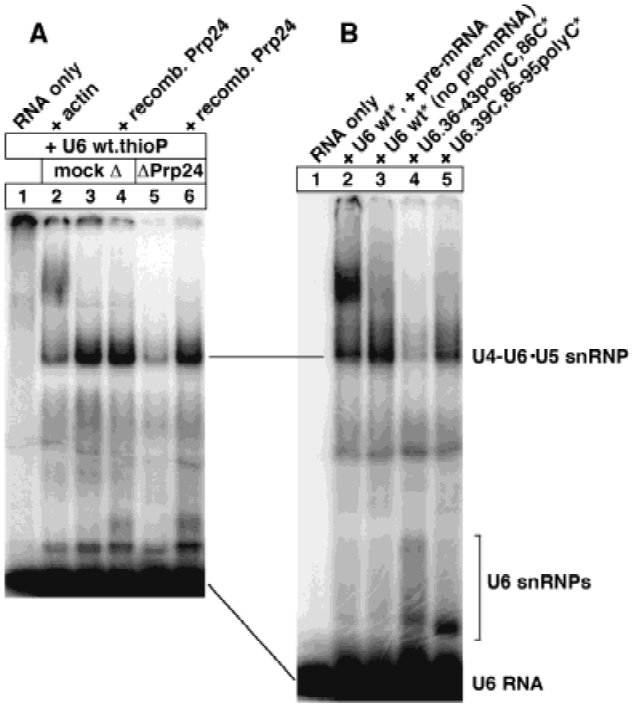

FIGURE 6. **A**: U4-U6•U5 snRNP assembly for U6-, Prp24-depleted yeast extract reconstituted with wild-type ^{32}P-labeled U6 RNA and treated or not with recombinant Prp24 protein. Control samples were mock depleted of endogenous Prp24 protein (lanes 2–4). Yeast splicing extract was treated with d1 oligonucleotide to digest endogenous U6 snRNA as described in Figure 2. Aliquots of the U6-depleted extract were incubated (lanes 5–6) or not (lanes 2–4) with 12CA5 antibody to endogenous epitope-tagged Prp24$(HA)_3$ protein in the extract. Aliquots of the mock- or Prp24-depleted, U6-depleted extract were treated with ^{32}P-labeled U6 RNA and assayed as in Figure 4B. For lane 2, actin pre-mRNA was added just prior to the 20-min incubation. **B**: U4-U6•U5 snRNP assembly for U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95) of yeast U6 RNA. Samples were prepared as in Figure 4B. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

of stably associated proteins comprising only Prp24 and the Lsm2–8 protein complex (Stevens et al., 2001). To assay binding of U6 snRNP proteins to our mutant U6 RNAs, we prepared yeast extracts that included an epitope tag on the sole copy of the Prp24 protein or on the sole copy of the Lsm4 protein (of the Lsm2–8 complex). These epitope-tagged extracts were reconstituted with a variety of individual U6 RNAs mutated in the 3′ terminal domain, the 3′ stem-loop, and/or the telestem sequences of U6 as listed in Table 3. Binding of the mutant U6 RNAs, carrying ^{32}P-radiolabels, to epitope-tagged Prp24 or Lsm4 protein was assayed by coimmunoprecipitation and scintillation counting of the bound U6 RNAs. This was followed by denaturing gel electrophoresis of the coimmunoprecipitated U6 RNAs and their supernatants to monitor and ensure the stability of U6 RNA in each sample (data not shown). The same ^{32}P-labeled U6 RNAs were also tested for their assembly into U6-containing snRNPs in vitro, including U4-U6•U5 tri-snRNPs, by using Raghunathan and Guthrie's (1998a) native gel electrophoresis system. Correlation of U6 RNA–protein binding affinity, U6 snRNP assembly profiles, and splicing activity for each mutation provides a more comprehensive understanding of the various roles of the 3′ domain, the 3′ stem-loop, and the telestem sequences of U6 snRNA.

The most obvious effect of mutating the upstream or downstream telestem sequence is that, under the immunoprecipitation conditions (150 mM NaCl), binding of the telestem mutant U6 RNAs to Prp24$(HA)_3$ was substantially diminished relative to such binding for wild-type U6 (Table 3). For the downstream telestem mutant, U6.39C,86–95polyC, this mutational disruption of the telestem also diminished Lsm protein binding, tri-snRNP assembly and splicing, all to 40–60% of wild-type U6 levels (Table 3; Fig. 3B, lanes 7 and 12; Fig. 6B, lane 4). In comparison to the downstream mutant, the upstream telestem mutant, U6.36–43polyC,86C, under parallel conditions showed ~1.5-fold less binding of Lsm proteins and ~1.6-fold less splicing activity than the downstream mutant, as well as a notable 3.5-fold reduction in the amount of tri-snRNP produced (Table 3; Fig. 6B, lane 3). For either upstream or downstream telestem mutants, the striking reduction in their binding of Prp24 protein (relative to wild-type U6) is consistent with the U6 snRNP assembly defects observed for these mutants by native gel electrophoresis: The telestem disruption mutations caused an accumulation of naked, mutant U6 RNA (Fig. 6B, lanes 3 and 4 as observed on a lighter exposure) that comigrated with synthetic U6 RNA alone on the same gel (not shown). The telestem mutations also caused an accumulation of free U6 snRNP complexes, including a complex that had unusually fast mobility on the gel (Fig. 6B, lanes 3 and 4) compared to mutant U6 snRNPs with a U80G or G81C mutation (cf. Fig. 6 in Ryan & Abelson, 2002). This accumulation of naked, mutant U6 RNA and mutant U6 snRNPs is most likely a result of diminished binding affinity for Prp24 protein that is necessary for U6 snRNP formation (Stevens et al., 2001) and for catalyzing U4-U6 snRNP assembly (see above; Raghunathan & Guthrie, 1998a). Also, the unusually fast mobility of some of the mutant telestem U6 snRNPs may reflect their incorporation of Lsm4 protein without also incorporating Prp24 protein, as suggested by the coimmunoprecipitation results for these same U6 telestem mutants (Table 3).

To further understand the effects of the 3′ terminal uridine tract mutations, we analyzed the abilities of these RNAs to form U6 snRNP, U4-U6, and U4-U6•U5 snRNPs by native polyacrylamide gel electrophoresis. We also tested their binding of Lsm core proteins as well as of Prp24 protein via coimmunoprecipitation experiments. Although we had found that mutation of the four U6 terminal uridines to polyadenine or polycytidine (U6.109–112polyA/polyC) had only a minor effect on

splicing yields, these mutations had a substantial effect (10-fold) on the binding of Lsm proteins (Table 3). This binding effect was expected, as studies of human U6 had shown that its terminal uridine tract was critical for binding to human Lsm proteins (Achsel et al., 1999). Surprisingly, our U6 uridine tract mutants under our same coimmunoprecipitation conditions were similarly inhibited for binding to Prp24 protein. This finding provides strong evidence for the suggestion from UV crosslinking and yeast two-hybrid studies that U6 binds Prp24 and Lsm proteins cooperatively (Vidal et al., 1999; Fromont-Racine et al., 2000). Furthermore, we found that U6 binding of Prp24 depends on or is strongly enhanced by U6 binding of Lsm proteins. Despite the reductions in binding of Lsm and Prp24 proteins to U6 RNA with 3′ uridine tract mutations, only minor effects were observed for U4-U6•U5 tri-snRNP assembly (Fig. 5B, lanes 4 and 5; Table 3), consistent with the minor effects on splicing found. Examination of the native gel showed some accumulation of the naked mutant U6 RNAs in the reconstituted samples relative to the wild-type U6 control (Fig. 5B, lanes 4 and 5 vs. lane 3).

When the remainder of the U6 3′ domain was mutated along with the 3′ terminal uridine tract (U6.86–112polyC and U6.86–108polyC.109–112polyA), U6 binding of Prp24 and Lsm4 proteins was diminished by 10- and 50-fold relative to wild-type U6 for each protein, respectively, and U4-U6•U5 tri-snRNP assembly and splicing were severely inhibited (Table 3; Fig. 4A, lanes 12 and 13; Fig. 5B, lanes 7 and 8). As expected for loss of Lsm and Prp24 protein binding to these 3′ domain mutants, the native gel showed substantial accumulations of naked mutant U6 RNAs not incorporated into U6 snRNPs (Fig. 5B, lanes 7 and 8). In contrast, polycytidine mutation of the entire 3′ domain except for the terminal uridine tract (U6.86–108polyC) showed a 12-fold higher normalized level of Lsm protein binding relative to that for mutation of the entire 3′ domain (U6.86–112polyC). This U6.86–108polyC mutant also showed a 2.5-fold higher normalized level of Lsm protein binding relative to that for mutation of only the terminal uridine tract (U6.109–112polyC or U6.109–112polyA)—its mutational complement for the 3′ domain (Table 3). These results confirm that the 3′ terminal uridine tract is a major determinant for binding to Lsm proteins in yeast U6. In contrast to the 2.5-fold higher normalized level observed for binding of Lsm proteins to U6.86–108polyC, binding of Prp24 protein to this mutant did not increase in a parallel fashion, presumably because mutation of U6 nt 86–108 includes mutation of nt 86–95, the 3′ sequence of the telestem, a sequence that is important for normal Prp24 binding (see above). This hindrance of Prp24 binding is consistent with an accumulation of naked mutant U6 RNA that was not incorporated into U6 snRNPs (Fig. 5B, lane 6). Our data suggest that although U6 binding of Prp24 protein depends on or is strongly enhanced by U6 binding of Lsm proteins, the binding of Lsm proteins is not dependent on U6 binding to Prp24. This point is most dramatically illustrated by the telestem disruption mutations, U6.36–43polyC,86C and U6.39C, 86–95polyC, which showed a substantial 25–50-fold reduction in binding of Prp24 protein relative to wild-type U6 RNA. In contrast, under the same conditions in vitro, these two telestem mutations showed a fairly modest 2–3-fold reduction in binding of Lsm proteins relative to wild-type U6 RNA (Table 3), demonstrating that Lsm protein binding is not dependent on U6 binding to Prp24 protein.

Compensatory mutations in the U6 telestem affect U6 RNA–protein binding, U4-U6•U5 tri-snRNP assembly, and pre-mRNA splicing in vitro

Our results demonstrated that mutational disruption of the entire U6 telestem blocks the normal binding of U6 RNA to Prp24 protein, whereas complete mutation of the upstream strand of the telestem also inhibits assembly of U4-U6•U5 tri-snRNP (Table 3). Despite these notable effects on Prp24 function, mutational disruption of the entire telestem can have a rather modest effect on splicing of two- to threefold (Table 3). If disruption of the telestem is a necessary event for spliceosome activation, then telestem disruption mutations are perhaps unlikely to produce substantial splicing defects. On the other hand, mutations that hyperstabilize the telestem helix might block spliceosome assembly and/or splicing and provide evidence for the presence and function of the U6 telestem in splicing, as has been observed for hyperstabilizing mutations in the U6 3′ stem-loop (Madhani et al., 1990; Wolff & Bindereif, 1993, 1995; Fortner et al., 1994). To test hyperstabilization of the telestem in vitro, we prepared U6 RNAs in which three contiguous A-U base pairs in the telestem were mutated to A-C mismatches or G-U wobble pairs or were fully transverted to G-C base pairs. These telestem mutants were U6.36–38polyC, U6.40–42polyG, U6.87–89polyC, U6.93–95polyG, and two potentially hyperstabilizing combinations, U6. 36–38polyC,93–95polyG, and U6.40–42polyG,87–89polyC.

In tandem experiments, we prepared a set of U6 telestem mutations that could restore telestem base pairing without hyperstabilization. In this set of constructs, three contiguous A-U base pairs in the telestem were mutated to A-A or U-U mismatches or were fully inverted to U-A base pairs. These telestem mutants were U6.36–38polyA, U6.40–42polyU, U6.87–89polyA, U6.93–95polyU and two potentially compensatory combinations, U6. 36–38polyA,93–95polyU, and U6.40–42polyU,87–89polyA.

The transverted and inverted telestem mutant U6 RNAs were assayed in U6-depleted extract for their

abilities to coimmunoprecipitate with epitope-tagged Prp24$(HA)_3$ protein in extract, to be assembled into U4-U6•U5 tri-snRNPs, and to reconstitute pre-mRNA splicing activity. The results are presented in Figure 7 and Table 4. Except for the telestem disruption mutations at nt 87–89, all other of our trinucleotide telestem-disruption mutations strongly inhibited binding of the mutant U6 RNA to epitope-tagged Prp24$(HA)_3$ protein in the coimmunoprecipitation assays, even though tri-snRNP and splicing were not inhibited or only mildly so (Table 4). However, the compensatory combinations that can restore base pairing in one-half of the telestem—that is, U6. 36–38polyC,93–95polyG and U6. 36–38polyA,93–95polyU—restored binding of U6 RNA to Prp24$(HA)_3$ protein. Indeed, the levels of these compensatory U6 RNAs coimmunoprecipitated by Prp24$(HA)_3$ was 2.5-fold greater than the level for wild-type U6 RNA. Therefore, the half of the telestem containing nt 36–38 and 93–95 (Fig. 1) is base paired in vitro and is tolerant of mutations that maintain base pairing. In contrast, the potentially compensatory combination in the other half of the telestem—that is, U6.40–42polyU,87–89polyA—did not restore the Prp24 binding defect of telestem disruption mutation U6.40–42poly U (Table 4).

Examination of the half of the telestem where sequences 40–42 and 87–89 are found (Fig. 1) revealed a striking difference between the 40–42 sequence and the 87–89 sequence when each was mutated and assayed for U6–Prp24 binding, tri-snRNP assembly and splicing (Table 4). Telestem disruption mutation U6.87–89polyC actually enhanced U6 RNA–Prp24 binding, and the comparable U6.87–89polyA mutation only very weakly diminished U6 RNA–Prp24 binding in coimmunoprecipitation assays. Consistent with these observations, the levels of tri-snRNP assembly and pre-mRNA splicing for each of these mutants were similar to their respective Prp24 coimmunoprecipitation levels. Hence,

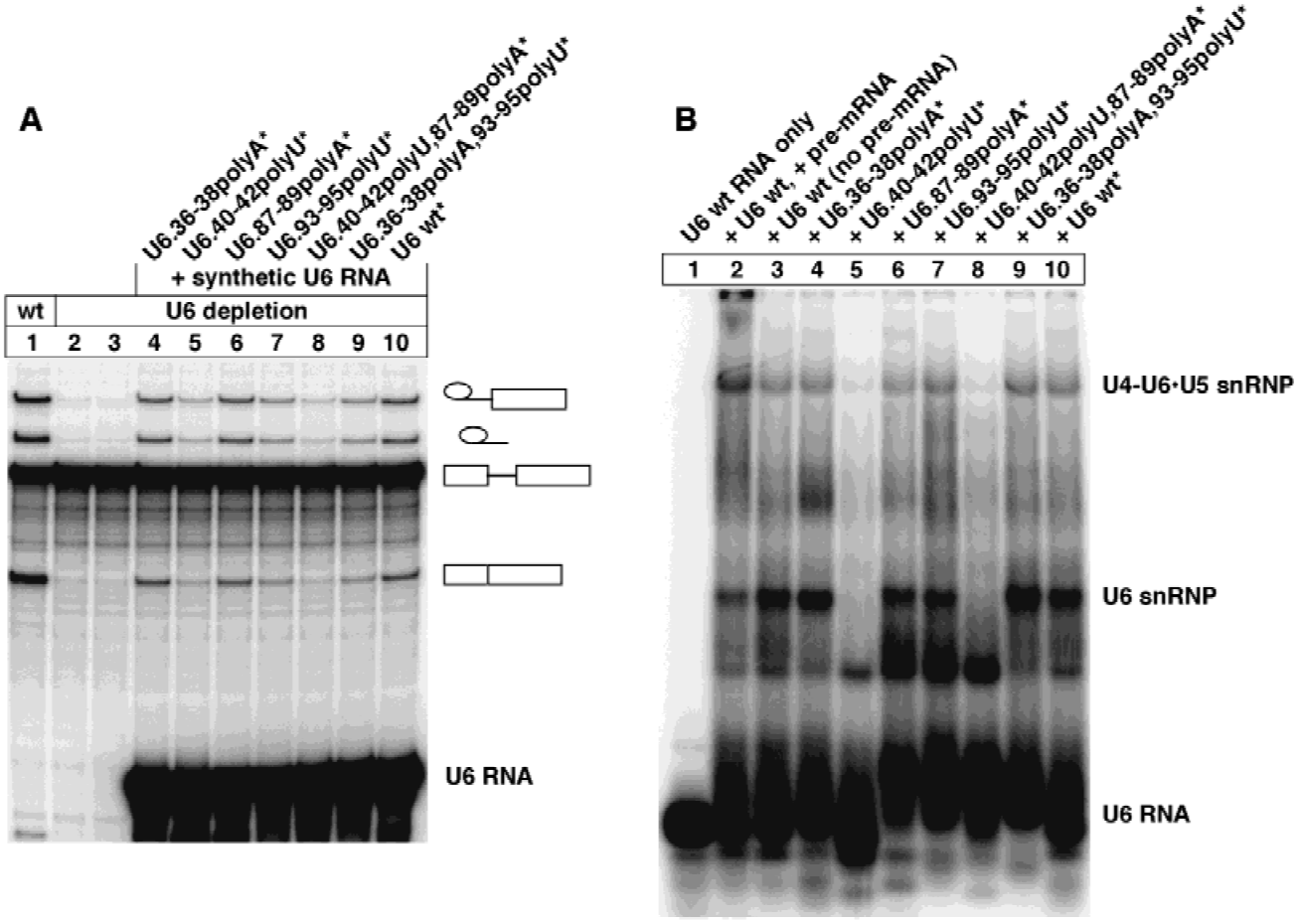

FIGURE 7. **A**: Splicing of ^{32}P-labeled actin pre-mRNA in U6-depleted yeast extract reconstituted with mutant and wild-type U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95) of yeast U6 RNA, including potentially compensatory mutations (lanes 8 and 9). Samples were prepared as in Figure 2. **B**: U4-U6•U5 snRNP assembly for U6-depleted yeast extract reconstituted with mutant and wild-type ^{32}P-labeled U6 RNAs. U6 mutations were introduced in the telestem sequences (nt 36–43 and 86–95). Samples were prepared as in Figure 4B except that 20 fmol of synthetic, ^{32}P-labeled U6 RNA was added as indicated. In synthetic U6 RNAs marked with an ∗, the last four 5′-3′ linkages at the 3′ ends were racemic phosphorothioates.

TABLE 4. Data summary for mutations in the 3′ telestem sequences of yeast U6 RNA.

Mutated U6 RNAs[a]	Splicing yields[b]	U4/U6-U5 yield[c,d]	Prp24(HA)$_3$ I.P.[d,e]
U6.36–38polyC	88%	139%	13%
U6.36–38polyA	86%	96%	3%
U6.93–95polyG	79%	51%	9%
U6.93–95polyU	35%	104%	5%
U6.36–38polyC,93–95polyG	49%	51%	248%
U6.36–38polyC,93–95polyU	—	—	4%
U6.36–38polyA,93–95polyU	35%	142%	245%
U6.36–38polyA,93–95polyG	—	—	2%
U6.40–42polyG	28%, 31%[f]	23%[g]	16%
U6.40–42polyU	19%	15%[g]	2%
U6.87–89polyC	102%, 114%[f]	105%	140%
U6.87–89polyA	77%	76%	82%
U6.40–42polyG,87–89polyC	8%, 9%[f]	5%[g]	—[h]
U6.40–42polyU,87–89polyA	9%	16%[g]	1%
U6 wild type	100%	100%	100%

[a]For all polyA- and/or polyU-mutated U6 RNAs and their U6 wild-type controls, the last 4-nt 5′-3′ linkages at the 3′ terminus are racemic phosphorothioates.

[b]Splicing yields for reconstituted, synthetic U6 RNAs are normalized relative to the yield for reconstituted, synthetic wild-type U6 RNA as a control.

[c]Yield of U4/U6-U5 tri-snRNP assembled from reconstituted U6 RNA, separated by native gel electrophoresis and normalized relative to the U6 wild-type control.

[d]Average yield of two or more trials.

[e]Relative amount of reconstituted U6 RNA coimmunoprecipitated with 12CA5 antibodies on protein A-Sepharose beads, normalized relative to the U6 wild-type control.

[f]Yield in a duplicate experiment.

[g]U4/U6-U5 tri-snRNP assembly gel showed aberrantly high levels of naked mutant U6 RNA not incorporated into U6 snRNPs.

[h]We were unable to generate sufficient quantities of U6.40–42polyG,87–89polyC RNA for this experiment.

in the half of the telestem containing nt 40–42 and 87–89, telestem base pairing is not important for Prp24 binding (or for splicing), in contrast to base pairing in the other half of the telestem. However, in contrast to mutations of nt 87–89, the mutations of nt 40–42 and the other trinucleotide telestem disruption mutations inhibited U6–Prp24 binding by at least sixfold in our assay. Indeed, the strongest inhibitions of U6 snRNP assembly, tri-snRNP assembly, and splicing were noted for trinucleotide mutations of nt 40–42 (Fig. 7). Therefore, the specific bases of nt 40–42 and not their base pairing status are important for Prp24 binding, U6 snRNP assembly, and splicing. In contrast, nt 87–89 in the same half of the telestem are fully tolerant of base mutations. Thus, our experiments provided no evidence that this half of the telestem is actually base paired at any time during spliceosome assembly or splicing.

Another result from our studies of trinucleotide telestem mutations is that, although nearly all of the telestem disruption mutations strongly inhibited U6–Prp24 coimmunoprecipitation, these mutations (except for those at nt 40–42; see above) showed at most a twofold effect on tri-snRNP assembly and up to a threefold effect on splicing. These effects are exactly parallel to those observed for U6.39C,86–95polyC (Table 3), which completely disrupted telestem base pairing. Taken together, these effects of telestem disruption on tri-snRNP assembly and pre-mRNA splicing are strikingly modest relative to other, previously reported U6 point mutations that inhibit pre-mRNA splicing (Fabrizio & Abelson, 1990; Ryan & Abelson, 2002).

DISCUSSION

Effects of truncating the 5′ or 3′ terminal domain of yeast U6 snRNA on splicing

In our ongoing effort to better understand the biochemical mechanism of pre-mRNA splicing, we investigated whether the 5′ and 3′ terminal domains of yeast U6 RNA (nt 1–46 and 86–112) are required for splicing in vitro. In HeLa nuclear extract, truncation of the entire 5′ domain of human U6 at a position that maintains only 3 nt upstream of the essential ACAGAGA sequence resulted in a low level of splicing activity (<10% of wild-type U6; Wolff & Bindereif, 1992). This demonstrated that the 5′ domain is not required for splicing in HeLa extract. Our truncation of the 5′ domain of yeast U6 maintains 8 nt upstream of the ACAGAG sequence, and we found that it gave a 23–31% yield of splicing products (relative to full-length U6), thus demonstrating that at least 80% of the 5′ domain of yeast U6 is similarly not required for splicing in yeast extract.

A 3′-truncated U6 RNA lacking the last 18 nt (U6.1–94) is able to reconstitute 34% of wild-type U6 splicing activity. However, a slightly further truncated U6 RNA (U6.1–90) was not viable in yeast (Bordonné & Guthrie, 1992). We were interested in determining the maximal 3′-end truncation of U6 that can support splicing in vitro. This information is useful for assessing the minimal chemical requirements for U6 RNA in splicing. We made two especially relevant observations. One is that U6.1–94 and shorter truncations appear to interfere with the normal degradation of excised intron lariats from spliced pre-mRNAs. Because it is known that the 3′ terminus is required for binding to Lsm proteins, one can imagine that the Lsm proteins may play a role in spliceosome disassembly and/or lariat degradation, perhaps in association with Prp43 and/or Prp22, both DEAH-box proteins required for dissociation of excised lariat and spliced mRNA, respectively, from yeast spliceosomes (Arenas & Abelson, 1997; Schwer & Gross, 1998; Wagner et al., 1998). Six of the seven U6-associated Lsm proteins, that is, Lsm2–7 proteins, form a different complex with Lsm1 and cytoplasmic factors to degrade mRNAs in the cytoplasm (Bouveret et al., 2000; Tharun et al., 2000). This suggests the possibility that perhaps some of the U6-associated Lsm proteins may recruit factors to degrade excised intron lariats in

yeast. The second notable observation is that truncation of the entire 3′ terminal domain, nt 86–112, is the largest 3′ truncation shown to support a detectable level of splicing activity in vitro. Hence, the entire 3′ terminal domain, including the U6 sequence of U2-U6 helix II, the U6 telestem, and an important binding domain for Lsm proteins, enhances the efficiency of splicing but is not necessary for in vitro splicing to occur. However, the backbone phosphate linkage at A79-U80 in the U6 3′ stem-loop is known to be essential for the first chemical step of splicing (Yean et al., 2000; Fabrizio & Abelson, 1992). Therefore, further truncations that would interfere with the proper functioning of this phosphate group are expected to block splicing.

Mutational analysis of the 3′ terminal domain of yeast U6 snRNA

Within the 3′ terminal domain of yeast U6 snRNA, nt 92–102 can base pair with the 5′ end of U2 snRNA to form U2-U6 helix II, and the presence of this helix in the 3′ terminal domain is conserved among eukaryotes. This helix was shown to be required for splicing in mammalian cells (Datta & Weiner, 1991; Wu & Manley, 1991). The yeast U6 3′ terminal domain is tolerant of multiple mutational substitutions as well as some internal deletions (Fabrizio et al., 1989; Madhani et al., 1990; Bordonné & Guthrie, 1992; Field & Friesen, 1996). In one report, transversion mutation of 9 of 11 nt of the U6 helix II sequence was lethal and could not be rescued by compensatory U2 mutations, whereas transversion mutation of all 11 nt of the complementary U2 strand was viable in yeast (although in vivo splicing was not assayed for possible defects; Field & Friesen, 1996). This demonstrated that U2-U6 helix II as usually represented is not essential in yeast. However, inspection of the yeast U2 sequence just downstream of helix II (see Fig. 1C of Ryan & Abelson, 2002) suggests that a close duplication of U2 nt 9–13 of helix II is present at nt 15–20, and this duplication could provide an alternative base pairing for a portion of helix II and thus a redundancy of function. This could also explain the asymmetry of mutational effects reported for U6 versus U2 strands of helix II (although we note that the U6 helix II sequence overlaps the 3′ telestem sequence). In all of the viable yeast U6 mutants previously reported, one can find remnants of helix II base pairing and possible alternative base pairings, suggesting that the function of helix II might be provided by a subset of U2-U6 base pairs in this region.

We examined the role of the U6 3′ terminal domain in splicing by making extensive mutational substitutions and deletions in a systematic manner. The helix II sequence of U6 contains only one cytidine, and the remaining 3′ terminal domain has only one additional cytidine; therefore we mutated the entire U6 helix II sequence to polycytidine. This mutant, U6.93–102polyC, reconstituted 35–45% of wild-type U6 splicing activity in vitro and was viable in yeast at all temperatures tested (16–37 °C). Therefore, in contrast to the lethal phenotype observed previously for mutation of 9 of 11 U6 nucleotides in helix II (Field & Friesen, 1996), we found that mutation of 10 of the 11 U6 helix II nucleotides is viable and decreases splicing efficiency by two- to threefold. The U6 helix II sequence (nt 92–102) overlaps the U6 telestem sequence of the 3′ terminal domain (nt 86–95) at nt 92–95. To separate the splicing effects of helix II disruption from those of telestem disruption, we mutated upstream telestem nt 36–38 to polycytidine or polyadenine to disrupt base pairing with complementary telestem nt 93–95. We found that the telestem mutants U6.36–38polyC and U6.36–38polyA reconstituted 88% and 86% of wild-type U6 splicing activity, respectively, and this effect (for disruption of the telestem portion overlapping helix II and perhaps some other unknown effects) should be kept in mind when considering the splicing yield measured for the helix II mutant U6.93–102polyC (found to be 35–45%). Though we have not demonstrated that our helix II U6 mutation can be suppressed by a compensatory base-pairing mutation in the U2 strand in vitro, it is known that helix II can form in yeast because the growth defects of helix II disruption mutations can be suppressed by compensatory base-pair restoring mutations (Field & Friesen, 1996). Our helix II mutant, U6.93–102polyC, is viable over a full range of temperatures (Table 2), so the total effects of helix II disruption in vivo cannot be very deleterious and are probably close to our in vitro measurements.

U6 telestem sequences are important for binding of U6 snRNA to Prp24 protein during U6 snRNP assembly

Polycytidine mutation of the entire upstream portion or the entire downstream portion of the U6 telestem was viable in yeast at all temperatures tested (16–37 °C) and produced a two- to threefold splicing defect in vitro (Table 3), similar to the effect we found for mutational disruption of U2-U6 helix II. These results show that disruption of the telestem has a relatively weak effect on splicing both in vitro and in vivo. Conversely, we found that mutation of the entire upstream or downstream portion of the telestem severely inhibited the association of these mutant U6 RNAs with Prp24 protein (Table 3), a component of free U6 snRNPs.

To explore the interaction between the U6 telestem and Prp24 protein in yeast, we first investigated whether Prp24 protein is active and important for spliceosome assembly in yeast extracts. During spliceosome assembly, naked yeast U6 snRNA is complexed by Lsm2–8 and Prp24 proteins to form free U6 snRNP (Stevens et al., 2001). The Prp24 component of free U6 snRNP is thought to be important for promoting duplex forma-

tion between free U6 and free U4 snRNPs to produce U4-U6 di-snRNP and U4-U6•U5 tri-snRNP complexes, at least for Prp24 protein in free U6 snRNPs recycled after a round of splicing (Raghunathan & Guthrie, 1998a). During the assembly of U4-U6•U5 tri-snRNP, Prp24 is dissociated from U6 snRNP or is much less tightly associated with it (Shannon & Guthrie, 1991; Gottschalk et al., 1999; Stevens & Abelson, 1999). Formation of U4-U6•U5 tri-snRNP was previously shown to be necessary for spliceosome assembly and splicing (Fetzer et al., 1997), and tri-snRNP can bind pre-mRNA to assemble spliceosomal complexes in the presence of ATP in vitro (Raghunathan & Guthrie, 1998a). However, it was not known whether Prp24 plays a role in the biogenesis of U6 snRNP and tri-snRNP, as studying this process was complicated by the predominance of fully formed and/or recycled U6-containing snRNPs in extract. In the experiments reported here, endogenous epitope-tagged Prp24$(HA)_3$ protein in yeast extract was immunodepleted, and this depletion diminished the assembly of ^{32}P-labeled U6 RNA into de novo tri-snRNPs to 14–16% of mock-depletion levels. Addition of recombinant Prp24 restored tri-snRNP assembly to 75–97% of mock-depletion levels in replicate assays (Fig. 6A). Endogenous U6 snRNA in these samples was RNase H digested prior to immunodepletion or mock depletion of Prp24 protein, thus to avoid codepletion of U6 snRNP-associated proteins, including Lsm2–8 proteins. Thus, we found that Prp24 is functional in catalyzing the assembly of naked U6 snRNA into U4-U6•U5 tri-snRNPs during snRNP biogenesis. Furthermore, this function is important for tri-snRNP assembly in vitro.

Continuing our exploration of the relationship between the U6 telestem and Prp24 protein, we constructed mutant U6 RNAs with mutations that disrupt telestem base pairings. We also constructed some with additional compensatory mutations that can restore base pairing. Of those that potentially restore telestem base pairing, there were two types of mutational combinations: one type in which A-U base pairs of the telestem were inverted to U-A pairs, and another type in which A-U base pairs were transverted to G-C pairs that may hyperstabilize the telestem. The base-pair restoring combinations did show a dramatic restoration of the binding interaction between mutant U6 RNA and Prp24$(HA)_3$ protein when the compensatory mutations were incorporated in the half of the telestem containing nt 36–39 and 92–95 (see Fig. 1), that is, when the compensatory mutants U6.36–38polyA,93–95polyU and U6.36–38polyC,93–95polyG were individually assayed for coimmunoprecipitation with Prp24$(HA)_3$ (Table 4). The compensatory effect for these two U6 mutants was 50-fold and 25-fold, respectively. Parallel controls corroborate that the compensatory effects were dependent on restoration of Watson–Crick base pairing rather than on various combinations of upstream and downstream telestem mutations. The control combinations, U6.36–38polyA,93–95polyG and U6.36–38polyC,93–95polyU, cannot restore telestem base pairing and did not suppress the Prp24 binding defects that were observed for each separate trinucleotide mutation (Table 4). These results reveal that base pairing in one half of the telestem, nt 36–39 and 92–95, occurs in vitro and is important for the normal binding of U6 snRNA to Prp24 protein. The same half of the U6 telestem was found to exist in vivo by compensatory mutational analysis (Vidaver et al., 1999). Our results confirm that this half of the telestem exists and is important for Prp24 function. The results also imply that U6–Prp24 binding is not particularly sensitive to the base-paired sequence of this half of the telestem. Thus, we determined that a general, base-paired RNA helix in the half of the telestem at nt 36–39,92–95 is important for binding of Prp24 to U6 snRNA.

In contrast, the other half of the putative telestem, nt 40–43 and 86–89, whose existence has not yet been supported by mutational analysis, does not lose binding affinity for Prp24 when its base pairs are disrupted via mutation. In fact, mutational disruption of this half of the telestem in U6.87–89polyC showed increased binding affinity for Prp24$(HA)_3$ protein as well as increased levels of U4-U6•U5 tri-snRNP assembly and pre-mRNA splicing (Table 4). This suggests that if the telestem forms at nt 40–43,86–89, then it may be disrupted to facilitate the binding of Prp24 protein. Not all mutations of U6 nt 86–89 enhanced Prp24 binding, tri-snRNP assembly, and/or splicing like the U6.87–89polyC mutation did. In fact, the effects of the equivalent polyA mutation, U6.87–89polyA, on these activities were very mildly inhibitory (Table 4). The difference between these polyC and polyA mutations may reflect that the 87–89polyC mutation involved replacing Us with Cs that maintained pyrimidines at nt 87–89, whereas the 87–89polyA mutation involved replacing the Us with purines, and this introduction of purines weakly inhibited the U6–Prp24 binding interaction.

Another important finding for the unconfirmed half of the telestem is that mutation of nt 40–42 causes a substantial loss of binding affinity between Prp24 protein and U6 RNA (Table 4). The mutations U6.40–42polyG and U6.40–42polyU caused the most substantial reductions in the levels of U6 snRNP assembly, tri-snRNP assembly, and splicing relative to our other trinucleotide telestem mutations (Figs. 4 and 7). These mutational defects were not relieved by introducing additional mutations that could potentially restore telestem base pairing, that is, for U6.40–42polyG,87–89polyC and U6.40–42polyU,87–89polyA. We conclude that the adenosine bases at nt 40–42 are especially important for U6 binding of Prp24 protein to form free U6 snRNP (Fig. 7, lanes 5 and 8). Therefore, the adenosines 40–42 as well as a short, nonspecific RNA helix at nt 36–38,93–95 are specifically important structural

elements of yeast U6 snRNA for Prp24 binding and function. A survey of U6 snRNA sequences among eukaryotes from yeast to humans shows that a eukaryotic consensus sequence that aligns with yeast adenosines 40–42 is RAR, in which R represents purine A or G. Furthermore, aside from budding yeasts, the RAR sequence in other organisms is not capable of base pairing with the equivalent sequence of yeast nt 87–89; therefore this half of the telestem cannot form in most eukaryotes as it can conceivably form in budding yeasts. This finding is consistent with our observation that base pairing within this half of the telestem is not important for U6 function. Although formation of the other half of the telestem helix (at nt 36–39,92–95) is important for U6–Prp24 binding under the conditions of the immunoprecipitation assay, no telestem base pairing is essential for yeast viability, tri-snRNP assembly, or pre-mRNA splicing as discussed above (see Table 2 and U6.39C,86–95polyC in Table 3). In contrast, Prp24 protein function is essential for yeast viability (Vijayraghavan et al., 1989). Therefore, the role of the U6 telestem in Prp24 function most likely contributes part, but not all, of the essential function of Prp24 protein in yeast. In addition to binding to U6 snRNA and catalyzing U4-U6 annealing, Prp24 is thought to interact with Lsm proteins in U6 snRNP (Fromont-Racine et al., 2000). Also, Prp24 has an undefined genetic interaction with Prp21 of U2 snRNP (Vaidya et al., 1996) that may be important for assembly of U1•U2•U4-U6•U5 penta-snRNPs during spliceosome assembly in yeast (Stevens et al., 2002).

Our proposal that Prp24 recognizes half of the telestem helix (at nt 36–39,92–95) and the three adenosine bases at nt 40–42 is consistent with previous studies of mutations in yeast U6 RNA. Bordonné and Guthrie (1992) replaced large segments of the yeast U6 gene with aligned human U6 sequences, and they monitored growth for these U6 chimeras in *S. cerevisiae*. They found that the entire 5′ domain and part of the central domain of yeast U6 (nt 1–53) can be replaced with the aligned human U6 sequence while maintaining growth at 18 °C and 30 °C. Similarly, replacing nt 87–108 of the yeast U6 3′ domain with the equivalent human U6 sequence supported growth at 18, 30, and 37 °C, whereas deletion of this sequence was lethal. This substitution of the 3′ domain is particularly interesting because the incorporated human sequence provides identical nucleotides to form the Prp24-recognized half of the telestem whereas the other (unconfirmed) half of the telestem cannot form in this case. Thus, these substitutions of the human U6 sequence into yeast do conform to the U6–Prp24 recognition elements that we have identified. If both the 5′ and 3′ domains of yeast U6 are simultaneously replaced by the human U6 sequences, the yeast cells are not viable (Bordonné & Guthrie, 1992). Similarly, human U6 RNA does not reconstitute splicing activity in U6-depleted yeast extract (Fabrizio et al., 1989). Conceivably, the simultaneous substitution into yeast of both the 5′ and 3′ domains of human U6 RNA, which are extensively complementary to each other, might form an extensively base-paired helix in yeast (as forms in human cells; Brow & Vidaver, 1995) that may not function sufficiently for splicing in yeast or yeast extract.

Our data strongly suggest that Prp24 may bind directly to nt 40–43 in free U6 snRNP, as outlined here. Jandrositz and Guthrie (1995) showed that proteinase K treatment of purified U6 snRNP causes nt 40–43 to become accessible to chemical modification. This demonstrated that these nucleotides are protected in U6 snRNP by a protein component, but it was unclear whether a protein binds directly to these nucleotides or whether a protein stabilizes an RNA–RNA interaction involving these nucleotides. As mentioned above, we found that affinity-purified yeast U6 snRNP contains only Prp24 and Lsm2–8 proteins. Based on our co-immunoprecipitation experiments, the most likely U6 snRNP protein to bind to nt 40–43 is Prp24 protein, as polycytidine mutation of nt 36–43 strongly inhibited U6 binding of Prp24 protein but not the binding of Lsm proteins (Table 3). The only other suggested candidate(s) for binding to nt 40–43 in free U6 snRNP are complementary nt 86–89 in the downstream U6 telestem sequence. In considering whether nt 40–43 might be protected from chemical modification by base pairing within the U6 telestem, we note that Jandrositz and Guthrie (1995) found that the lone guanosine of nt 40–43 and 86–89 (i.e., G86) is accessible to chemical modification, whereas its potential telestem partner, C43, is shielded from modification within the 40–43 sequence. This result argues against protection of nt 40–43 by telestem base pairing and suggests that these nucleotides are likely protected by direct binding to Prp24 protein. Additional experiments are needed to confirm and elucidate this.

In summary, our data suggest that specific structural features of the U6 telestem are important for binding of Prp24 protein to U6 snRNA. These structural features are the general RNA helix at nt 36–38,93–95 as well as the three adenosine bases at nt 40–42. It was previously shown that the identity of A91 in the U6 telestem is important for U6-Prp24 binding (Shannon & Guthrie, 1991). We found that Prp24 is important for the assembly of naked U6 RNA into U4-U6•U5 tri-snRNP in vitro. Therefore, the specified features of the telestem are important for U6–Prp24 binding during U6 snRNP assembly. However, the telestem is not necessary in yeast or in yeast extract for relatively high levels of tri-snRNP assembly and splicing to occur (see U6.39C,86–95polyC in Table 3). This is because Prp24 protein need not bind to U6 snRNA with wild-type affinity to assemble the U6 RNA into near wild-type levels of functional tri-snRNP for splicing (see also examples U6.36–38polyA/C and U6.93–95polyG in Table 4). The iden-

tified features of the telestem clearly enhance the binding affinity between U6 RNA and Prp24 protein in free U6 snRNP, but this binding enhancement is generally not necessary for tri-snRNP assembly and splicing. The enhancement can become necessary if U6–Lsm protein binding affinity is diminished, as by mutating the 3′ terminal uridine tract of U6 snRNA (nt 109–112). For example, one can see that the U6.86–108polyC mutant cannot form the telestem and had 10-fold weaker binding affinity for Prp24 protein in the coimmunoprecipitation experiments (relative to wild-type U6 RNA; Table 3). In contrast, the splicing activity for this mutation was reduced by only threefold but dropped to trace levels when U6–Lsm binding affinity was simultaneously diminished by mutating the 3′ terminal uridine tract as well (cf. U6.86–108polyC versus U6.86–108polyC,109–112polyA and U6.86–112polyC; Table 3). Below, we discuss the reciprocal situation, that is, loss of U6–Lsm binding affinity has a relatively mild effect on tri-snRNP assembly and splicing unless U6–Prp24 binding affinity is also diminished.

In contrast to the telestem helix, the consensus RAR sequence at telestem nt 40–42 is very important not only for U6–Prp24 binding but also for tri-snRNP assembly, presumably at the Prp24-dependent step(s) of tri-snRNP assembly, as outlined here. As the RAR sequence is conserved from yeast to humans, this may be an important recognition motif for the function of Prp24 homologs in various organisms. The RAR sequence was largely protected against hydroxyl radical footprinting when pure, transcribed yeast U6 RNA was bound to recombinant Prp24 protein (Ghetti et al., 1995). Nucleotides on either side of this sequence were protected as well, including most of those in the 5′ telestem sequence. (The 3′ telestem sequence was not assayed in the Prp24 footprinting assay.) Our results also relate to previous chemical modification assays of the chemical accessibility of U6 nucleotides in free U6 snRNP versus U4-U6 di-snRNP (Jandrositz & Guthrie, 1995). In purified wild-type U6 snRNP, nt 40–43 were not accessible to chemical modification, whereas they were accessible in purified wild-type U4-U6 di-snRNP. Mutant U4.G14C-U6 di-snRNP differed from the wild-type di-snRNP in that nt 40–43 of U6 RNA in the mutant complex were protected from chemical modification and in that the mutant complex remained bound to Prp24 protein (Shannon & Guthrie, 1991). Another distinction of the mutant U4.G14C-U6 di-snRNP was that each testable U6 nucleotide from U64 through G86 was accessible to chemical modification, demonstrating that this region of U6 was not base paired to the mutant U4 snRNA nor was it base paired intramolecularly to form a U6 3′ stem-loop (at nt 63–84) as found in free U6 snRNP and mature spliceosomes (Fig. 1; see also Fig. 1C in Ryan & Abelson, 2002). This suggested that perhaps Prp24 protein remains bound to U6 nt 40–43 in nascent U4-U6 snRNPs following the unwinding of the U6 3′ stem-loop but before the unwound region is fully base paired with U4 snRNA to form mature U4-U6 di-snRNP, which no longer binds Prp24 protein (Shannon & Guthrie, 1991; see also Fig. 3 of Raghunathan & Guthrie, 1998a, for samples containing ATP which were actively producing nascent U4-U6 snRNP). Our results are fully consistent with this model and provide substantial evidence for it. We partially purified the U4-U6 di-snRNP from yeast in sufficient quantities to allow silver staining of its protein components that had been separated by SDS-PAGE. We could clearly see protein bands consistent with the presence of the Lsm proteins (associated with U6 RNA), the Sm proteins (associated with U4 RNA), and the Prp3-Prp4 heterodimer (Snu13 may be present as it overlaps the SmE and Lsm8 bands; Gottschalk et al., 1999); however, no trace of any protein was found in or near the region of Prp24 protein migration (our unpubl. results). We know from our previous work that Prp24 protein is visible by silver staining of proteins separated from purified free U6 snRNP (Stevens et al., 2001). These results are consistent with the above model in that mature wild-type U4-U6 snRNP is not bound to Prp24 protein.

Combining our data with the known information about the secondary structure of U6 snRNA leads to a new secondary structure as presented in Figure 1. The confirmed half of the U6 telestem (nt 36–38,93–95) adjoins the consensus sequence RAR (nt 40–42) to provide recognition motifs that we have identified as important for binding of U6 snRNA to Prp24 protein during assembly of U6 snRNP. The current data suggests that the unconfirmed half of the telestem (nt 40–43,86–89) may not be base paired in free U6 snRNP; therefore we represent this possible helix with dashed lines. The consensus RAR sequence at nt 40–42 is highlighted because it is more important for U6 snRNP assembly than any of the other telestem sequences (see Fig. 7B). We propose that the RAR sequence binds directly to Prp24 protein in free U6 snRNP, based on our results and those of Jandrositz and Guthrie (1995) and Ghetti et al. (1995). The U6 sequence at nt 44–62 is depicted in our model as having an undefined structure, even though nt 54–59 have been presented in previous reports as base paired with nt 29–34 in free U6 snRNP (Vidaver et al., 1999). However, such base pairing is not consistent with results from chemical modification studies of purified wild-type U6 snRNP (Jandrositz & Guthrie, 1995). Therefore, we prefer to represent nt 26–35 and 44–62 as having undefined secondary structure.

An interesting observation is apparent by contrasting the splicing activities of U6.93–95polyG and U6.93–95polyU. Relative to the 93–95polyG mutation, the 93–95polyU mutation shows an accumulation of mutant U4-U6•U5 tri-snRNP and a corresponding reduction in pre-mRNA splicing. Therefore, the U6.93–95polyU

mutant-containing tri-snRNP is apparently deficient in its ability to assemble into spliceosomes. Based on the current understanding of spliceosome assembly, tri-snRNP associates with the 5′ splice site in the assembling spliceosome, and U2 and U6 snRNAs form U2-U6 helixes I and II as U4 snRNA dissociates from base pairing with U6 snRNA. The U6.93–95polyU mutation is part of the U6 telestem and U2-U6 helix II at different times (see Fig. 1 of Ryan & Abelson, 2002). The 93–95polyU mutation cannot base pair with its intended tri-uridine partner in the U6 telestem or that in U2-U6 helix II, in contrast to the equivalent 93–95polyG mutation, which can form wobble pairs with both of these tri-uridine sequences. As U2-U6 helix II is thought to form when tri-snRNP binds U2 snRNA during spliceosome assembly, the most likely scenario is that the 93–95polyU mutant is deficient in forming U2-U6 helix II, and this defect caused the observed accumulation of mutant tri-snRNP and the corresponding reduction in splicing. Mutation of the entire U6 sequence of U2-U6 helix II to polycytidine showed an equivalent splicing defect (see U6.93–102polyC in Table 2). A recent study supports the idea that U2-U6 helix II can form when the U6 RNA strand is concurrently base paired with U4 RNA during spliceosome assembly (Xu & Friesen, 2001). However, the subsequent unwinding of the U4-U6 RNA duplex in forming active spliceosomes does not require the presence or formation of U2-U6 helix II (see results for U6.93–102polyC in Table 2). The U4-U6 RNA duplex is unwound via a process mediated by ATP and Brr2 protein, a DExH-box ATPase (Raghunathan & Guthrie, 1998b). It is possible that the telestem re-forms when the U4-U6 RNA duplex is unwound at this stage. Our results neither support nor rule out this possibility.

Our data are also compatible with the possibility that the U6 telestem may play a more important role in the splicing pathway other than to promote U6–Prp24 binding in free U6 snRNP, albeit a role that cannot be essential for yeast viability, as formation of the telestem is not essential for growth at viable temperatures (16–37 °C). Perhaps the telestem forms after completion of a round of splicing to help release U6 snRNP from the spliceosome for recycling. One can imagine that formation of the entire telestem (nt 36–43,86–95) may help stabilize nascent U6 snRNA transcripts prior to their association with Prp24 protein in the assembly of U6 snRNPs. Further experiments are necessary to test for additional functions of the U6 telestem.

In light of a tantalizing proposal that Prp24 protein may play a role in spliceosome assembly after U4-U6 duplex formation (Vidaver et al., 1999), several results must be considered. Raghunathan and Guthrie (1998a) found that immunodepletion of >97% of epitope-tagged Prp24 protein in yeast extract had no effect on the ability of preformed, endogenous tri-snRNP to bind pre-mRNA and promote wild-type levels of splicing products. However, the amount of Prp24 needed for a possible catalytic step is expected to be substantially lower than the stoichiometric requirements for Prp24 in U6 snRNP formation and recycling. In considering the original data that suggested a role for Prp24 in spliceosome assembly after U4-U6 di-snRNP assembly, a few questions come to bear. For instance, the difference between the levels of U4-U6 annealing for U6 A62G versus A62U,C85A shown in Figure 7A of Vidaver et al. (1999), which was an important experimental basis for the proposal, varies from the difference measured for the same mutations in a previous report (reported as “data not shown” on p. 225 of Fortner et al., 1994). A similar variation is apparent when comparing the levels of annealed U4-U6 mutant snRNP versus free U4 snRNP for the U6 A62G mutant at 30 °C shown in Figure 7A of Vidaver et al. (1999) in contrast to that shown in Figure 2D of Fortner et al. (1994) for the same U6 mutation and conditions. When we performed such U4-U6 annealing assays, our control samples showed that gel-purified U4 and U6 RNA transcripts were annealed to a significant extent in the standard 1× hybridization buffer of the assay (Li & Brow, 1993), without addition of any yeast extract or protein to these controls (data not shown). Therefore, we used the native gel electrophoresis method of Raghunathan and Guthrie (1998a) to assay the native levels of assembled U4-U6 di-snRNP, as described above. The question of a role for Prp24 after U4-U6 snRNP assembly remains quite interesting and merits further study.

Binding of yeast U6 RNA to Prp24 protein is dependent on or is strongly enhanced by U6 binding of Lsm proteins

The short uridine tract at the 3′ terminus of human U6 snRNA was shown to be an essential determinant for U6 binding to Lsm proteins in human spliceosomes. By similarity, the tract of four uridines at the 3′ end of yeast U6 RNA is expected to be an essential determinant of Lsm binding in yeast as well. In yeast, the last 18 nt at the 3′ end of U6 were shown to be necessary but not sufficient for binding to Lsm proteins (Vidal et al., 1999). Interestingly, this 3′ truncated yeast U6 RNA was able to reconstitute 34% of full-length U6 splicing activity in vitro (Fabrizio et al., 1989, and Table 1, part A). Mutation of the 3′ terminal uridine tract only, to polyadenine or polycytidine, was found to substantially inhibit U6 binding of Lsm proteins, as predicted (see U6.109–112polyA/C, Table 3). However, these mutations exerted only minor effects on tri-snRNP assembly and splicing (Fig. 5; Table 3). This is because the U6-associated Lsm proteins need not bind to U6 snRNA with wild-type affinity in order to assemble the U6 RNA into near wild-type levels of functional tri-snRNP for splicing. The 3′ terminal uridine tract clearly enhances the binding affinity between U6 RNA and Lsm4 protein

in free U6 snRNP, but this binding enhancement is generally not necessary for tri-snRNP assembly and splicing. The enhancement can become necessary if U6–Prp24 binding affinity is diminished, as by mutating the U6 telestem as well. For example, one can see that the U6.109–112polyA/C mutations diminished U6–Lsm4 binding affinity by ~10-fold in the coimmunoprecipitation experiments (relative to wild-type U6 RNA; Table 3). In contrast, the splicing activity for this mutation was only slightly diminished, but dropped to trace levels when U6–Prp24 binding affinity was simultaneously diminished by mutating the remainder of the 3′ domain (cf. U6.109–112polyA/C vs. U6.86–108polyC, 109–112polyA and U6.86–112polyC; Table 3). We conclude that functional U6 snRNP can tolerate losses of U6–Prp24 binding affinity or U6–Lsm core binding affinity but not losses of both simultaneously, at least not in our examples. Presumably, the protein–protein interactions between Prp24 and Lsm proteins in U6 snRNP (Fromont-Racine et al., 2000) can moderate a loss in U6–Lsm or U6–Prp24 binding affinity.

The most surprising effect of mutating only the 3′ terminal uridine tract of U6 was the effect on its binding to Prp24 protein. Mutation of the 3′ uridine tract to polyadenine or polycytidine caused a substantial reduction in binding to Prp24 protein, parallel to the reduction in binding to Lsm proteins. Previously, it was found that truncation of the last 18 nt of yeast U6 resulted in loss of crosslinking to Prp24 and Lsm proteins as well as the loss of crosslinking to many U6-proximal proteins (Vidal et al., 1999). We had found that truncation of the last 18 nt of yeast U6 (i.e., U6.1–94) results in degradation of this RNA in yeast extracts to shorter lengths matching U6.1–91/90 as well as shorter, and none of the original U6.1–94 was found in the yeast extract at 23 °C after 30 min (shorter time points were not assayed). This finding suggested to us that the U6.1–94 truncation, and especially its U6.1–91/90 degradation products that form in extracts, might impinge on the function of the downstream sequence of the U6 telestem (nt 86–95), which we found to be important for the normal binding of Prp24 protein to U6 RNA. Therefore, such large 3′ end truncations are expected to inhibit U6 binding of Prp24 in extract, as observed by Vidal et al. (1999). Our mutations of only the four 3′ terminal uridines inhibited not only binding of Lsm proteins, as predicted, but also binding of Prp24 protein to a parallel extent under the same conditions (Table 3), without any truncation, significant degradation (Fig. 5A), or additional mutation of the U6 RNA. This result strongly suggests that U6 binding to Prp24 protein depends on or is strongly enhanced by U6 binding to the Lsm core proteins.

In contrast, extensive mutation of the upstream or downstream portions of the U6 telestem inhibited U6 binding of Prp24 protein but had a substantially smaller effect on binding of Lsm proteins under parallel conditions (Table 3). Therefore, U6 binding to Lsms does not appear to depend on binding to Prp24. The 3′ terminal uridine tract of nascent U6 transcripts is initially associated with the yeast La protein, Lhp1, and the 3′ end becomes phosphorylated at the 3′ hydroxyl during U6 snRNP assembly in vivo (Lund & Dahlberg, 1992). In this assembly pathway, Lhp1 is thought to hand off the U6 transcript to the Lsm proteins (Pannone et al., 2001). Our findings suggest that Prp24 protein can then readily bind to the assembling U6 snRNP. The primary effect of U6–Lsm binding on the binding of Prp24 to the U6–Lsm complex may derive from a kinetic effect, that is, perhaps generating the proper U6 substrate for Prp24 binding is greatly accelerated by U6–Lsm binding. Such effects might predominate over the gain in free energy generated by the interaction between Prp24 and Lsm proteins (Fromont-Racine et al., 2000). Alternatively, if the primary effect of Lsm binding is thermodynamic, U6's association with the Lsm core may provide a substantially more stable binding site for Prp24 than that provided by U6 RNA alone, which was measured to be ~100 nM for gel-purified U6 transcript and recombinant Prp24 protein in aqueous buffer (Ghetti et al., 1995). Further experiments are necessary to determine these kinetic and thermodynamic parameters.

In summary, the 3′ terminal uridine tract of yeast U6 RNA is important for U6 binding to Lsm proteins as expected. U6 binding to Prp24 protein is dependent on or is greatly enhanced by U6 binding to Lsm proteins, suggesting an order to U6 snRNP assembly. U6-Prp24 binding is also dependent on half of the U6 telestem and the adjoining RAR sequence. However, some essential functions of Prp24 do not depend on the presence of the U6 telestem, as the telestem is not essential for growth. Truncation of the entire 3′ terminal domain or nearly the entire 5′ terminal domain of yeast U6 allows for detectable levels of splicing to proceed in vitro; hence we have redefined the minimal functional group requirements of yeast U6 snRNA in active spliceosomes. Our results also contribute to a revised model of the yeast U6 snRNA secondary structure (Fig. 1).

MATERIALS AND METHODS

Procedures for the syntheses of U6 RNAs, for reconstitutions of U6 snRNA in yeast extract, and for in vitro splicing assays are described in our companion paper (Ryan & Abelson, 2002).

Generation of polycytidine mutations in U6 plasmids for in vivo experiments

PolyC mutant U6 plasmids were created by a two-step PCR mutagenesis procedure. Briefly, an oligonucleotide primer incorporating a *Bam*HI restriction endonuclease site 300 nt upstream of the U6 transcription start site was used in a PCR reaction with another oligonucleotide incorporating the mutation(s) of choice. In a separate PCR reaction, an oligonu-

cleotide primer incorporating a *Sal*I restriction endonuclease site 300 nt downstream of the U6 3′ end was used in a PCR reaction with another oligonucleotide that is the reverse complement of the corresponding mutation. Isolated PCR products were combined and subjected to a PCR reaction containing only the extant oligonucleotide primers. Gel-purified PCR products of the correct size were isolated, were restriction endonuclease digested with *Bam*HI and *Sal*I, and were ligated into *Bam*HI- and *Sal*I-digested pRS414 (Christianson et al., 1992).

Isolation and characterization of mutant U6 plasmids from *S. cerevisiae*

For the two strains containing polycytidine U6 mutations that yielded a subpopulation of slow-growing colonies on 5-FOA media, we recovered the U6 plasmids from these colonies to check for spontaneous, intragenic U6 suppressor mutations as follows. Selected colonies were resuspended in YPD medium and grown to an optical density of ~2.0 at 600 nm. Cells were harvested by centrifugation, washed once with 1 M sorbitol, and digested in 1 M sorbitol containing 10 mg/mL lyticase (Sigma) for 30 min at 23 °C. Spheroplasts were collected by gentle centrifugation (4,000 × *g*) and washed twice with 1 M sorbitol. Spheroplasts were disrupted and plasmids were harvested with a kit for bacterial plasmid purification (Qiagen). The resulting plasmid DNA was transformed into DH10B *E. coli* (Hanahan, 1983). U6 snRNA genes were sequenced using oligonucleotide primers corresponding to sequences at 100 nt upstream of the U6 snRNA transcription start site and at 100 nt downstream of the last nucleotide of the U6 gene on the plasmid.

U4-U6•U5 tri-snRNP assembly and immunodepletion of Prp24 protein

To assay the assembly of U4-U6•U5 tri-snRNPs in vitro, nondenaturing polyacrylamide gel electrophoresis was performed as described by Raghunathan and Guthrie (1998a). We prepared high-specific activity, synthetic U6 RNAs as described (Ryan & Abelson, 2002), except that one internal oligonucleotide piece of U6 was 5′-phosphorylated with 4 moleequivalents of [γ-^{32}P]ATP (>7,000 Ci/mmol; ICN) and no cold ATP, whereas the other internal ligation sites were 5′-phosphorylated as a group with 1 mM ATP before combining the oligonucleotide pieces of U6 RNA during phenol extraction. Wild-type yeast extract was reconstituted with synthetic, high-specific-activity ^{32}P-labeled U6 RNA as described (Ryan & Abelson, 2002) and incubated at 23 °C for 20 min, then put on ice. For depletion of both endogenous U6 snRNA and Prp24 protein, 20 μL of epitope-tagged Prp24(HA)$_3$ extract for each sample was first depleted of endogenous U6 snRNA as usual and then incubated with 0.5 μL of 12CA5 antibody (5 μg/μL; Boehringer Mannheim) or phosphate-buffered saline on ice for 1 h. This was added to buffer D-washed, protein A-Sepharose beads (20 μL of a 0.1 g/mL suspension in water; Pharmacia) and nutated with periodic flicking for 1.5 h at ~4 °C. Synthetic, ^{32}P-labeled U6 RNA was added and incubated as above. Some samples were also treated with 0.1 μg of recombinant Prp24 protein and incubated at 23 °C for an additional 30 min, then put on ice. One microliter of nondenaturing gel loading buffer (see above) was added to samples prior to loading onto the native gel.

Coimmunoprecipitation of U6 RNA bound to epitope-tagged Prp24 or Lsm4 protein

Epitope-tagged Prp24(HA)$_3$ extract (10 μL per sample) was reconstituted with synthetic, high-specific-activity U6 RNA as above for U4-U6•U5 tri-snRNP assembly in wild-type extract. After the 20-min incubation, any U6 RNA bound to epitope-tagged Prp24 protein was coimmunoprecipitated by first incubating the individual samples with 1.1 μL of 0.45 μg/μL 12CA5 antibody mixed with 2.7 U/μL of Prime RNase inhibitor (Eppendorf) for 1 h on ice. For each immunoprecipitation, 15 μL of protein A-Sepharose beads (0.1 g/mL suspension in water) were washed three times with 300 μL of IPP$_{150}$ buffer (10 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 0.05% NP-40 in DEPC-treated water), and 250 μL of IPP$_{150}$ were used to rinse the extract-antibody sample into the tube of washed beads that was nutated at ~4 °C overnight (~12 h). Beads were spun at 2,000 × *g* (5,000 rpm in Eppendorf centrifuge) to remove supernatants, and the beads were washed three times with 300 μL of IPP$_{150}$ on ice. Coimmunoprecipitated, radiolabeled U6 RNA was quantitated by both scintillation counting and PhosphorImager quantitation of the eluted U6 RNA on a denaturing polyacrylamide gel. For coimmunoprecipitation of reconstituted U6 RNA bound to Lsm4 protein in epitope-tagged Lsm4 extract, the above protocol was followed, except immunoprecipitations were conducted using polyoma antibody precoupled to protein G-Sepharose beads as previously described (Stevens, 2000).

Northern blots of native gels for U4-U6•U5 tri-snRNP or spliceosome assembly

To prepare blots, the nondenaturing gel was transferred to Whatman paper (as a support) and placed against Zeta-probe membrane (BioRad) for electroblotting as described by Fabrizio et al. (1989). Prehybridization and hybridization were also carried out exactly as described, except we used sheered and boiled salmon sperm DNA and different northern probes. The U6 probe was 5′-end-labeled dU6.30–112 DNA oligonucleotide. U1, U2, U4, U5, and actin pre-mRNA probes were prepared by random priming of hexameric DNA oligonucleotides annealed to gel-purified PCR products of the respective genes.

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REFERENCES

Achsel T, Brahms H, Kastner B, Bachi A, Wilm M, Lührmann R. 1999. A doughnut-shaped heteromer of human Sm-like proteins binds to the 3′-end of U6 snRNA, thereby facilitating U4-U6 duplex formation in vitro. *EMBO J 18*:5789–5802.

Arenas JE, Abelson JN. 1997. Prp43: An RNA helicase-like factor involved in spliceosome disassembly. *Proc Natl Acad Sci USA 94*:11798–11802.

Boeke JD, Trueheart J, Natsoulis G, Fink GR. 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular-genetics. *Methods Enzymol 154*:164–175.

Bordonné R, Guthrie C. 1992. Human and human-yeast chimeric U6 snRNA genes identify structural elements required for expression in yeast. *Nucleic Acids Res 20*:479–485.

Bouveret E, Rigaut G, Shevchenko A, Wilm M, Séraphin B. 2000. An Sm-like protein complex that participates in mRNA degradation. *EMBO J 19*:1661–1671.

Brow DA, Guthrie C. 1988. Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. *Nature 334*:213–218.

Brow DA, Vidaver RM. 1995. An element in human U6 RNA destabilizes the U4/U6 spliceosomal RNA complex. *RNA 1*:122–131.

Burge CB, Tuschl T, Sharp PA. 1999. Splicing of precursors to mRNAs by the spliceosomes. In: Gestland RF, Cech TR, Atkins JF, eds. *The RNA world*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 525–560.

Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene 110*:119–122.

Collins BM, Harrop SJ, Kornfeld GD, Dawes IW, Curmi PIG, Mabbutt BC. 2001. Crystal structure of a heptameric Sm-like protein complex from archaea: Implications for the structure and evolution of snRNPs. *J Mol Biol 309*:915–923.

Datta B, Weiner AM. 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. *Nature 352*:821–824.

Fabrizio P, Abelson J. 1990. Two domains of yeast U6 small nuclear RNA required for both steps of nuclear precursor messenger RNA splicing. *Science 250*:404–409.

Fabrizio P, Abelson J. 1992. Thiophosphates in yeast U6 snRNA specifically affect pre-mRNA splicing in vitro. *Nucleic Acids Res 20*:3659–3664.

Fabrizio P, McPheeters DS, Abelson J. 1989. In vitro assembly of yeast U6 snRNP: A functional assay. *Genes & Dev 3*:2137–2150.

Fetzer S, Lauber J, Will CL, Lührmann R. 1997. The [U4/U6•U5] tri-snRNP-specific 27K protein is a novel SR protein that can be phosphorylated by the tri-snRNP-associated protein kinase. *RNA 3*:344–355.

Field DJ, Friesen JD. 1996. Functionally redundant interactions between U2 and U6 spliceosomal snRNAs. *Genes & Dev 10*:489–501.

Fortner DM, Troy RG, Brow DA. 1994. A stem/loop in U6 RNA defines a conformational switch required for pre-mRNA splicing. *Genes & Dev 8*:221–233.

Fromont-Racine M, Mayes AE, Brunet-Simon A, Rain J-C, Colley A, Dix I, Decourty L, Joly N, Ricard F, Beggs JD, Legrain P. 2000. Genome-wide protein interaction screens reveal functional networks involving Sm-like proteins. *Yeast 17*:95–110.

Ghetti A, Company M, Abelson J. 1995. Specificity of Prp24 binding to RNA: A role for Prp24 in the dynamic interaction of U4 and U6 snRNAs. *RNA 1*:132–145.

Gottschalk A, Neubauer G, Banroques J, Mann M, Lührmann R, Fabrizio P. 1999. Identification by mass spectrometry and functional analysis of novel proteins of the yeast [U4/U6•U5] tri-snRNP. *EMBO J 18*:4535–4548.

Hanahan D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol 166*:557–580.

Hastings ML, Krainer AR. 2001. Pre-mRNA splicing in the new millennium. *Curr Opin Cell Biol 13*:302–309.

Jandrositz A, Guthrie C. 1995. Evidence for a Prp24 binding site in U6 snRNA and in a putative intermediate in the annealing of U6 and U4 snRNAs. *EMBO J 14*:820–832.

Kambach C, Walke S, Young R, Avis JM, de la Fortelle E, Raker VA, Lührmann R, Li J, Nagai K. 1999. Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs. *Cell 96*:375–387.

Kandels-Lewis S, Séraphin B. 1993. Role of U6 snRNA in 5′ splice site selection. *Science 262*:2035–2039.

Kim CH, Abelson J. 1996. Site-specific crosslinks of yeast U6 snRNA to the pre-mRNA near the 5′ splice site. *RNA 2*:995–1010.

Lesser CF, Guthrie C. 1993. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science 262*:1982–1988.

Li Z, Brow DA. 1993. A rapid assay for quantitative detection of specific RNAs. *Nucleic Acids Res 21*:4645–4646.

Lund E, Dahlberg JE. 1992. Cyclic 2′,3′ phosphates and nontemplated nucleotides at the 3′ end of spliceosomal U6 small nuclear RNAs. *Science 255*:327–330.

Madhani HD, Bordonné R, Guthrie C. 1990. Multiple roles for U6 snRNA in the splicing pathway. *Genes & Dev 4*:2264–2277.

Madhani HD, Guthrie C. 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell 71*:803–817.

Madhani HD, Guthrie C. 1994. Randomization-selection analysis of snRNAs in vivo: Evidence for a tertiary interaction in the spliceosome. *Genes & Dev 8*:1071–1086.

Mayes AE, Verdone L, Legrain P, Beggs JD. 1999. Characterization of Sm-like proteins in yeast and their association with U6 snRNA. *EMBO J 18*:4321–4331.

McPheeters DS. 1996. Interactions of the yeast U6 RNA with the pre-mRNA branch site. *RNA 2*:1110–1123.

McPheeters DS, Abelson J. 1992. Mutational analysis of the yeast U2 snRNA suggests a structural similarity to the catalytic core of group I introns. *Cell 71*:819–831.

Mura C, Cascio D, Sawaya MR, Eisenburg DS. 2001. The crystal structure of a heptameric archaeal Sm protein: Implications for the eukaryotic snRNP core. *Proc Natl Acad Sci USA 98*:5532–5537.

Nilsen TW. 1998. RNA–RNA interactions in nuclear pre-mRNA splicing. In: Simons RW, Grunberg-Manago M, eds. *RNA structure and function*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 279–307.

Pandolfi D, Rauzi F, Capobianco ML. 1999. Evaluation of different types of end-capping modifications on the stability of oligonucleotides toward 3′- and 5′-exonucleases. *Nucleosides Nucleotides 18*:2071–2091.

Pannone BK, Kim SD, Noe DA, Wolin SL. 2001. Multiple functional interactions between components of the Lsm2-Lsm8 complex, U6 snRNA, and the yeast La protein. *Genetics 158*:187–196.

Raghunathan PL, Guthrie C. 1998a. A spliceosomal recycling factor that reanneals U4 and U6 small nuclear ribonucleoprotein particles. *Science 279*:857–860.

Raghunathan PL, Guthrie C. 1998b. RNA unwinding in U4/U6 snRNPs requires ATP hydrolysis and the DEIH-box splicing factor Brr2. *Curr Biol 8*:847–855.

Ryan DE, Abelson J. 2002. The conserved central domain of yeast U6 snRNA: Importance of U2-U6 helix I_a in spiceosome assembly. *RNA 8*:997–1010.

Schwer B, Gross C. 1998. Prp22, a DExH-box RNA helicase, plays two distinct roles in yeast pre-mRNA splicing. *EMBO J 17*:2086–2094.

Shannon KW, Guthrie C. 1991. Suppressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-binding motifs. *Genes & Dev 5*:773–785.

Sontheimer EJ, Steitz JA. 1993. The U5 and U6 small nuclear RNAs as active site components of the spliceosome. *Science 262*:1989–1996.

Stevens SW. 2000. Analysis of low-abundance ribonucleoprotein particles from yeast by affinity chromatography and mass spectrometry microsequencing. *Method Enzymol 318*:385–398.

Stevens SW, Abelson J. 1999. Purification of the yeast U4/U6•U5 small nuclear ribonucleoprotein particle and identification of its proteins. *Proc Natl Acad Sci USA 96*:7226–7231.

Stevens SW, Barta I, Ge HY, Moore RE, Young MK, Lee TD, Abelson J. 2001. Biochemical and genetic analyses of the U5, U6, and U4/U6•U5 small nuclear ribonucleoproteins from *Saccharomyces cerevisiae*. *RNA 7*:1543–1553.

Stevens SW, Ryan DE, Ge HY, Moore RE, Young MK, Lee TD, Abelson J. 2002. Composition and functional characterization of the yeast spliceosomal penta-snRNP. *Mol Cell 9*:31–44.

Tharun S, He W, Mayes AE, Lennertz P, Beggs JD, Parker R. 2000. Yeast Sm-like proteins function in mRNA decapping and decay. *Nature 404*:515–518.

Törö I, Thore S, Mayer C, Basquin J, Séraphin B, Suck D. 2001. RNA binding in an Sm core domain: X-ray structure and functional analysis of an archaeal Sm protein complex. *EMBO J 20*:2293–2303.

Vaidya VC, Seshadri V, Vijayraghavan U. 1996. An extragenic suppressor of prp24-1 defines genetic interaction between PRP24 and PRP21 gene products of *Saccharomyces cerevisiae*. *Mol Gen Genet 250*:267–276.

Valadkhan S, Manley JL. 2001. Splicing-related catalysis by protein-free snRNAs. *Nature 413*:701–707.

Vidal VPI, Verdone L, Mayes AE, Beggs JD. 1999. Characterization of U6 snRNA-protein interactions. *RNA 5*:1470–1481.

Vidaver RM, Fortner DM, Loos-Austin LS, Brow DA. 1999. Multiple functions of *Saccharomyces cerevisiae* splicing protein Prp24 in U6 RNA structural rearrangements. *Genetics 153*:1205–1218.

Vijayraghavan U, Company M, Abelson J. 1989. Isolation and characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*. *Genes & Dev 3*:1206–1216.

Wagner JDO, Jankowsky E, Company M, Pyle AM, Abelson JN. 1998. The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes. *EMBO J 17*:2926–2937.

Wassarman DA, Steitz JA. 1992. Interactions of small nuclear RNAs with precursor messenger RNA during in vitro splicing. *Science 257*:1918–1925.

Wolff T, Bindereif A. 1992. Reconstituted mammalian U4-U6 snRNP complements splicing: A mutational analysis. *EMBO J 11*:345–359.

Wolff T, Bindereif A. 1993. Conformational changes of U6 RNA during the spliceosome cycle: An intramolecular helix is essential both for initiating the U4-U6 interaction and for the first step of splicing. *Genes & Dev 7*:1377–1389.

Wolff T, Bindereif A. 1995. Mutational analysis of humanU6 RNA: Stabilizing the intramolecular helix blocks the spliceosomal assembly pathway. *Biochim Biophys Acta 1263*:39–44.

Wu J, Manley JL. 1991. Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. *Nature 352*:818–821.

Xu D, Friesen JD. 2001. Splicing factor Slt11p and its involvement in formation of U2/U6 helix II in activation of the yeast spliceosome. *Mol Cell Biol 21*:1011–1023.

Yean S-L, Wuenschell G, Termini J, Lin R-J. 2000. Metal-ion coordination by U6 small nuclear RNA contributes to catalysis in the spliceosome. *Nature 408*:881–884.