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The yeast Prp3 protein is a U4/U6 snRNP protein necessary for integrity of the U4/U6 snRNP and the U4/U6.U5 tri-snRNP

JAMES G. ANTHONY,*[1] ELAINE M. WEIDENHAMMER,*[2] and JOHN L. WOOLFORD, JR.
Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, USA

ABSTRACT

Previously, yeast *prp3* mutants were found to be blocked prior to the first catalytic step of pre-mRNA splicing. No splicing intermediates or products are formed from pre-mRNA in heat-inactivated *prp3* mutants or *prp3* mutant extracts. Here we show that Prp3p is a component of the U4/U6 snRNP and is also present in the U4/U6.U5 tri-snRNP. Heat inactivation of *prp3* extracts results in depletion of free U6 snRNPs and U4/U6.U5 tri-snRNPs, but not U4/U6 snRNPs or U5 snRNPs. Free U4 snRNP, normally not present in wild-type extracts, accumulates under these conditions. Assays of in vivo levels of snRNAs in a *prp3* mutant revealed that amounts of free U6 snRNA decreased, free U4 snRNA increased, and U4/U6 hybrids decreased slightly. These results suggest that Prp3p is required for formation of stable U4/U6 snRNPs and for assembly of the U4/U6.U5 tri-snRNP from its component snRNPs. Upon inactivation of Prp3p, spliceosomes cannot assemble from prespliceosomes due to the absence of intact U4/U6.U5 tri-snRNPs. Prp3p is homologous to a human protein that is a component of U4/U6 snRNPs, exemplifying the conservation of splicing factors between yeast and metazoans.

Keywords: pre-mRNA splicing; Prp3p; U4/U6 snRNP protein; *S. cerevisiae*; spliceosome assembly

INTRODUCTION

Splicing of introns from nuclear pre-mRNA in yeast and mammals occurs in a protein–RNA complex called the spliceosome, which contains the U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and a number of non-snRNP protein factors. Both the assembly and activation of the spliceosome are dynamic processes involving interactions among pre-mRNA, snRNPs, and various protein splicing factors (reviewed in Rymond & Rosbash, 1992; Hodges et al., 1993; Moore et al., 1993; Legrain & Chanfreau, 1994; Newman, 1994; Nilsen, 1994; Ares & Weiser, 1995; Guthrie, 1996; Krämer, 1996). Spliceosome assembly occurs through an ordered pathway, which has been determined by analyzing the kinetics of formation of intermediate complexes in vitro. First, the U1 snRNP assembles with the 5′ splice site of pre-mRNA to form the commitment complex; this interaction directs pre-mRNA into the splicing pathway. Subsequently, U2 snRNA base pairs with the intron branchpoint sequence to form the prespliceosome. The U5 snRNP associates with the U4/U6 snRNP to form a U4/U6.U5 tri-snRNP that assembles with the prespliceosome to form the mature spliceosome.

Activation of the spliceosome occurs through several conformational rearrangements of the snRNPs. The disruption of U4:U6 base pairing and the subsequent release of U4 snRNA from the spliceosome leaves U6 snRNA free to base pair with both the U2 snRNA and the 5′ splice site. In addition, the U5 snRNA associates with sequences in the 5′ exon, near the 5′ splice site. Thus, the 5′ splice site and the branchpoint adenosine are juxtaposed to facilitate step 1 of splicing. Before the second catalytic reaction of splicing, U5 snRNA base pairs with the 3′ exon near the 3′ splice site, to align the two exons for ligation. The splicing cycle is completed by release of snRNAs from the spliced intron and reassembly of U4/U6 and U4/U6.U5 snRNPs.

During this cycle of spliceosome assembly, pre-mRNA splicing, and spliceosome disassembly, a number of snRNP–snRNP and snRNP–pre-mRNA contacts are

Reprint requests to: John L. Woolford, Jr., Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Avenue, Pittsburgh, Pennsylvania 15213, USA; e-mail: jw17@andrew.cmu.edu.
*The first two authors contributed equally to the work.
[1]Present address: Digene Corp, Silver Spring, Maryland 20904-1966, USA.
[2]Present address: Department of Medicine, Division of Infectious Diseases, University of California, San Diego, San Diego, California 92093, USA.

established. Many of these interactions occur via RNA–RNA base pairing; however, a large number of protein factors are also required for splicing to occur. Each of the snRNPs shares eight common core or "Sm" proteins, essential for snRNP biogenesis. In addition, several proteins specifically associated with the U1, U2, U5, U6, U4/U6, or U4/U6.U5 snRNPs have been identified in yeast and mammals (reviewed in Lührmann et al., 1990; Krämer, 1996). Although the exact function of many splicing factors remains to be established, protein–protein and protein–RNA interactions are thought to be important for assembly and stability of snRNPs, and for establishing and altering snRNA and snRNP conformations and associations during the spliceosome cycle. Characterization of the roles of snRNP proteins may shed some light on the dynamics of snRNP assembly and function in splicing.

In the yeast *Saccharomyces cerevisiae*, more than 40 different proteins necessary for pre-mRNA splicing have been identified by genetic schemes, including screens for mutants defective in splicing, selections for suppressors of mutations in introns or splicing factors, or screens for mutants synthetically lethal with mutations in snRNAs or other splicing protein factors (reviewed in Ruby & Abelson, 1991; Rymond & Rosbash, 1992; Hodges et al., 1993; Legrain & Chanfreau, 1994). Among these, the *prp3* mutant was identified in a screen of temperature-sensitive yeast strains defective for RNA synthesis (Hartwell, 1967; Hartwell et al., 1970). Subsequent analysis revealed that *prp3* strains are temperature-sensitive (Ts^-) for pre-mRNA processing. Unspliced pre-mRNA accumulates in vivo and in vitro, implicating a role for Prp3p in assembly of the spliceosome or in catalysis of the first cleavage reaction (Rosbash et al., 1981; Larkin & Woolford, 1983; Lustig et al., 1986).

Previously, we isolated the *PRP3* gene to assess the function of Prp3p (Last et al., 1984). Antibodies raised against Prp3p were used to localize this protein to the yeast nucleus (Last & Woolford, 1986). Several observations suggested that Prp3p interacts with the U4/U6-specific snRNP protein Prp4p. Mutations in the *PRP4* gene are suppressed by extra copies of *PRP3* (Last et al., 1987); *prp3 prp4* double mutants exhibit synthetic lethality (Ruby et al., 1993; Maddock et al., 1994); and the *spp41* and *spp42* mutants, isolated as extragenic suppressors of *prp4* Ts^- mutations, suppress the Ts^- phenotype of strains bearing *prp3* mutations (Maddock et al., 1994). In this paper, we demonstrate that Prp3p is a component of the U4/U6 snRNP and is also present in U4/U6.U5 tri-snRNP particles. Assembly of spliceosomes from prespliceosomes is blocked in *prp3* mutants, most likely an indirect effect of disruption of U4/U6.U5 snRNP assembly in the absence of functional Prp3p. The failure to maintain the integrity of U6-containing snRNP particles in *prp3* mutants results in susceptibility of U6 snRNA to nucleolytic digestion. Prp3p is homologous to a human protein that is a component of the U4/U6 snRNP (Lauber et al., 1997), underscoring the conservation of splicing factors from yeast to man.

RESULTS

Prp3p is required for assembly of spliceosomes from prespliceosomes

The block of splicing reactions in *prp3* mutants prior to the first cleavage-ligation event both in vivo and in vitro (Rosbash et al., 1981; Larkin & Woolford, 1983; Lustig et al., 1986) suggests that Prp3p may play a role in spliceosome formation. Alternatively, or in addition, Prp3p may be required to promote the first step of splicing after the spliceosome has formed, similar to Prp2p and Spp2p (Roy et al., 1995a; Kim & Lin, 1996). To determine which, if any, spliceosomal complexes form in extracts derived from a *prp3* strain, we utilized nondenaturing gel electrophoresis to resolve prespliceosomes from spliceosomes (Arenas & Abelson, 1993). Splicing reactions were performed with *prp3-1* extracts at the indicated time after addition of radiolabeled template. A portion of the reaction was quenched and subjected to electrophoresis. Unheated *prp3-1* extracts formed complexes identical to those seen in extracts derived from a wild-type strain (Fig. 1A, cf. lanes 3–6 with 11 and 12). At early time points, prespliceosomes were observed; subsequently, the more slowly migrating complete spliceosome was detected. These results are consistent with the splicing activity of this extract (Fig. 1B, lane 2). In *prp3-1* extracts that were subjected to heat treatment prior to substrate addition, prespliceosomes formed; however, fully assembled spliceosomal complexes were not detected (Fig. 1A, lanes 7–10), nor were splicing products or intermediates generated (Fig. 1B, lane 3). These results suggest that Prp3p is involved in spliceosome formation, particularly in the formation of spliceosomes from prespliceosomes.

Prp3-1 mutant affects tri-snRNP assembly or stability

The failure of the U4/U6.U5 tri-snRNP to assemble with prespliceosomes to form spliceosomes in *prp3* mutant extracts might result indirectly from a defect in U4/U6 snRNP assembly or stability, or from failure of the U4/U6 snRNP to associate with the U5 snRNP to form a stable U4/U6.U5 tri-snRNP. Alternatively, Prp3p may play a direct role in tethering the tri-snRNP with prespliceosomes, as observed for Prp31p (Weidenhammer et al., 1997). To examine the effects of the *prp3-1* mutation on snRNP complexes, glycerol gradient fractionation of splicing extracts was performed. Extracts derived from a *PRP3* wild-type strain or a *prp3-1* strain were layered onto 10–30% glycerol gradients, and snRNP particles were separated by centrifugation and

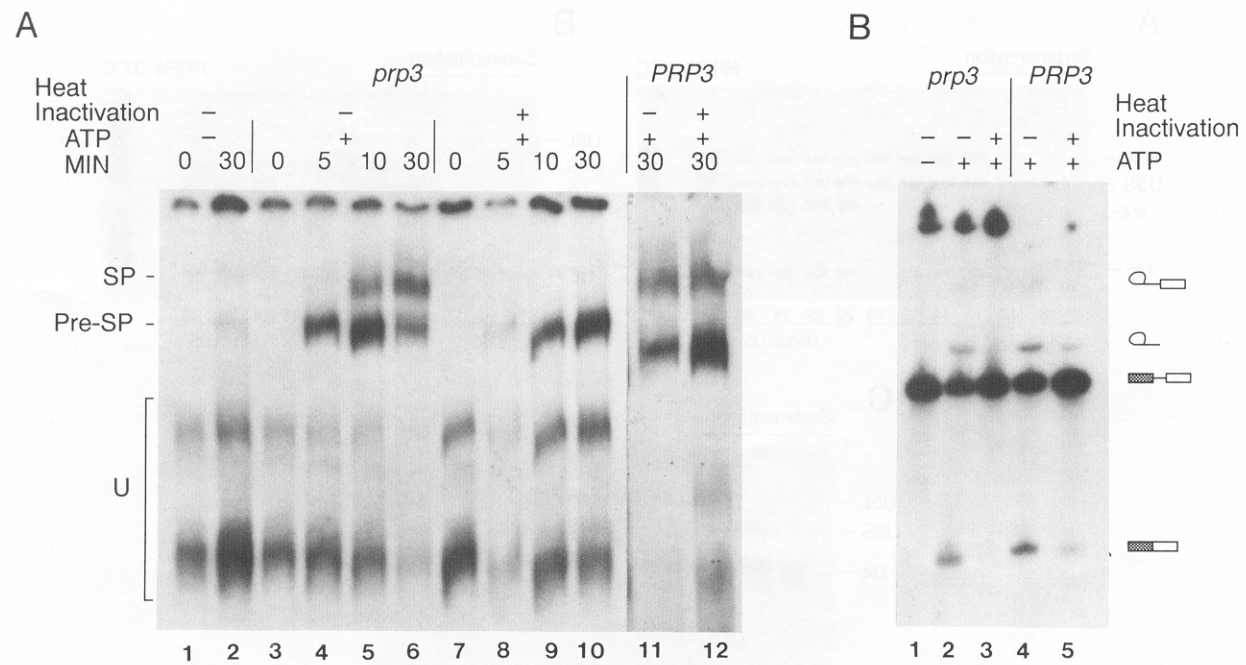

FIGURE 1. Prespliceosomes, but not spliceosomes, form in a heat-inactivated *prp3* extract. **A:** Extracts derived from *prp3-1* mutant strain JWY630 or wild-type *PRP3* strain JWY2878 were incubated at 37 °C (lanes 7–10, 12) or maintained on ice for 30 min. Radiolabeled transcript was then added and reactions were allowed to proceed for the indicated amount of time. Formation of splicing complexes was monitored by subjecting the samples to electrophoresis on a nondenaturing polyacrylamide gel. SP, mature spliceosome; Pre-SP, prespliceosome; U, nonspecific pre-mRNA complexes. **B:** A portion of each splicing assay was subjected to electrophoresis on a denaturing acrylamide gel to monitor formation of splicing intermediates and products. ▬○▭, pre-mRNA; ○▭, lariat intron-3′ exon intermediate; ○—, lariat intron product; ▬▭, spliced mRNA.

fractionated for RNA analysis. Resolution of U6, U4/U6, U5, and U4/U6.U5 snRNPs from a *PRP3* wild-type extract is shown in Figure 2A. Incubation of the *prp3-1* extract at 37 °C prior to fractionation resulted in a pattern different from wild type (Fig. 2C). No free U6 snRNP was detected, consistent with the reduced levels of U6 snRNA in *prp3-1* strains (Blanton et al., 1992, and see below). Additionally, a novel species containing U4 snRNA was present (fractions 9, 11). These same fractions also contained a faster migrating form of U4 snRNA, which might result from partial degradation of U4 snRNA upon inactivation of Prp3p. Some U4/U6 snRNP complexes were apparent (fractions 13, 15), as were free U5 snRNPs (fractions 15–21), but no U4/U6.U5 tri-snRNPs were detected. Although some free U5 snRNPs are detected in the fractions from the bottom of the gradient, no U4 or U6 snRNA was present in these samples. In contrast, no significant change in the amounts of each snRNP was observed upon incubating wild-type *PRP3* extracts at 37 °C (Fig. 2B). The depletion of U6 snRNPs and U4/U6.U5 tri-snRNPs, along with the accumulation of the novel U4 snRNP, supports the hypothesis that Prp3p is required for efficient assembly or stability of the U4/U6.U5 tri-snRNP complex, and further suggests a role in assembly or stabilization of the U6 and U4/U6 snRNPs.

Prp3-1 mutants exhibit altered assembly or stability of the U4/U6 snRNP

To examine the effect of the *prp3-1* mutant protein on U4/U6 assembly or stability in vivo, nondenaturing gels were used to differentiate U4/U6 duplexes from free U4 and free U6 snRNAs (Fortner et al., 1994). RNA was extracted from cells that were grown to mid-log phase at the permissive temperature and either maintained at 23 °C or shifted to 37 °C for varying amounts of time. Following annealing of radiolabeled oligomers complementary to U4 and U6 snRNAs, the complexes were separated by nondenaturing gel electrophoresis. Unlike a wild-type *PRP3* strain, the *prp3-1* strain accumulated free U4 snRNA upon shifting to the nonpermissive temperature; this defect was also observed to some extent at 23 °C (Fig. 3). In addition, levels of the U6 snRNA decreased rapidly in the *prp3-1* strain and U4/U6 hybrids appeared to be somewhat destabilized upon shifting to 37 °C. Quantification with an Ambis PhosphorImager, using the small nucleolar RNA snR190 as a loading control, confirmed these observations. These data indicate that Prp3p is required to efficiently assemble and maintain the integrity of the U6 and U4/U6 snRNP particles.

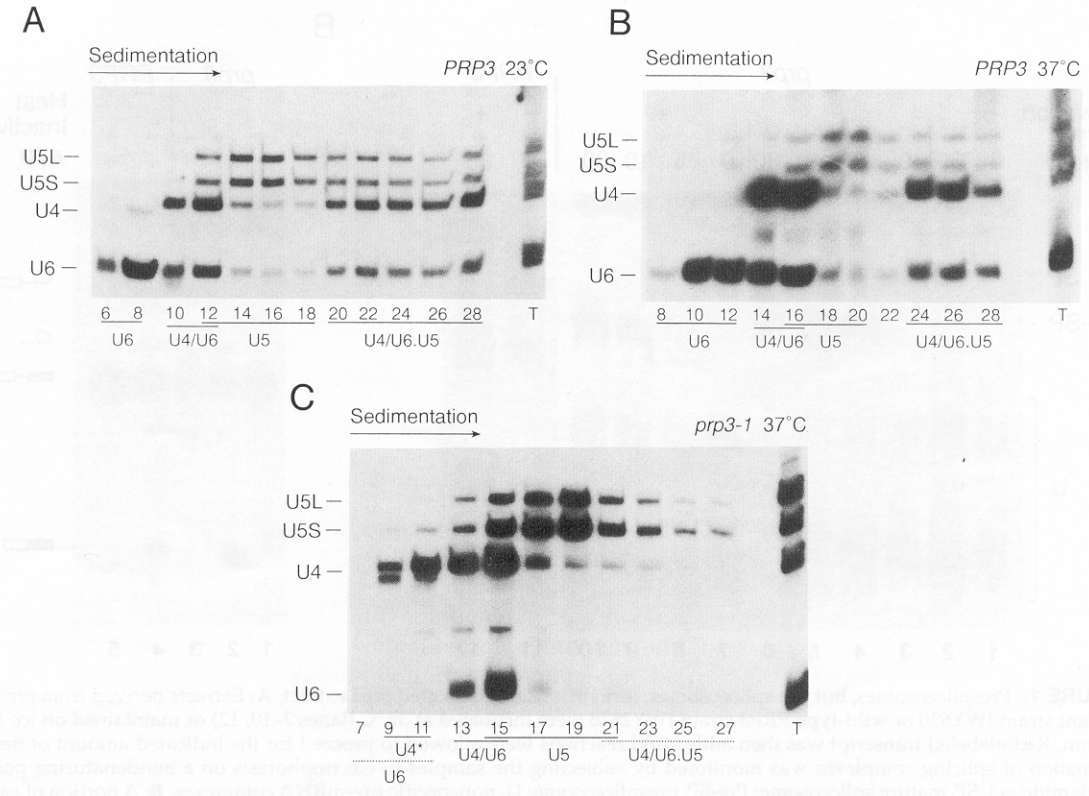

FIGURE 2. Heat-inactivation of *prp3* extracts affects the stability of snRNP complexes. Glycerol gradient fractionation of snRNP particles in extracts derived from wild-type *PRP3* strain JWY2878 (**A,B**) or from *prp3-1* mutant strain JWY630 (**C**). Extracts were incubated at 37 °C for 30 min (B,C) or maintained on ice for this period (A); samples were layered onto 10–30% glycerol gradients and snRNP complexes were separated by centrifugation. RNA was derived from even- or odd-numbered gradient fractions and subjected to northern analysis using radiolabeled oligonucleotides complementary to the U4, U5, and U6 snRNAs. Note the presence of a free U4 snRNP in fractions 9 and 11 of the heat-inactivated *prp3-1* extract, and the absence of a free U6 snRNP in these same fractions (compared with fractions 8–12 of the heated *PRP3* extract, shown in B). Also note the lack of tri-snRNP complexes in fractions from the bottom of the gradient (fractions 23–27).

Prp3p is associated specifically with U4/U6 snRNPs and is present in U4/U6.U5 tri-snRNPs

To test whether Prp3p is a component of yeast spliceosomal snRNPs, co-immunoprecipitation experiments were performed. Antibodies against Prp3p co-immunoprecipitated the U4, U5L, U5S, and U6 snRNAs from splicing extracts of wild-type yeast (Fig. 4, lanes 2, 3, and 8). No precipitation of the U1 or U2 snRNAs was detected with anti-Prp3p antiserum, although these snRNAs could be immunoprecipitated with anti-trimethylguanosine antibodies (Fig. 4, lane 7). The precipitation of U4, U5, and U6 snRNAs in these experiments was specific to antibodies directed against Prp3p. No RNAs were precipitated with pre-immune serum (Fig. 4, lane 6) or with anti-Prp3p antiserum blocked by pre-incubation with TrpE-Prp3p fusion protein (Fig. 4, lanes 4 and 5). Pre-treatment of anti-Prp3p with TrpE protein alone did not prevent immunoprecipitation of the RNAs (Fig. 4, lanes 2 and 3).

The U4 and U6 snRNAs are bound together in a single U4/U6 snRNP by base pairing between the RNAs (Rinke et al., 1985; Siliciano et al., 1987; Brow & Guthrie, 1988). The U4, U5, and U6 snRNPs can be detected in splicing extracts in a single U4/U6.U5 tri-snRNP complex (Cheng & Abelson, 1987; Lossky et al., 1987; Lamond et al., 1988; Bordonné et al., 1990). U5 and U6 are also present individually in U5 or U6 snRNPs (Cheng & Abelson, 1987; Bordonné et al., 1990; Shannon & Guthrie, 1991). The immunoprecipitation results described above suggest that Prp3p is part of the tri-snRNP, either as an integral component of the U4/U6 or U5 snRNP, or as a tri-snRNP specific protein. To determine with which of these snRNPs Prp3p is associated specifically, we assayed the efficiency of co-immunoprecipitation of the snRNAs by anti-Prp3p antiserum in the presence of increasing concentrations of NaCl. Addition of high concentrations of salt to yeast splicing extracts results in dissociation of the U4/U6.U5 complex to individual U4/U6 and U5 snRNPs (Cheng & Abelson, 1987; Banroques & Abelson, 1989; Abovich et al., 1990; Behrens & Lührmann, 1991). Immunoprecipitation of U4, U5, and U6 by anti-Prp3p was not affected significantly by concentrations of NaCl up to 400 mM in the splicing extracts, although incubation at higher concentrations of NaCl

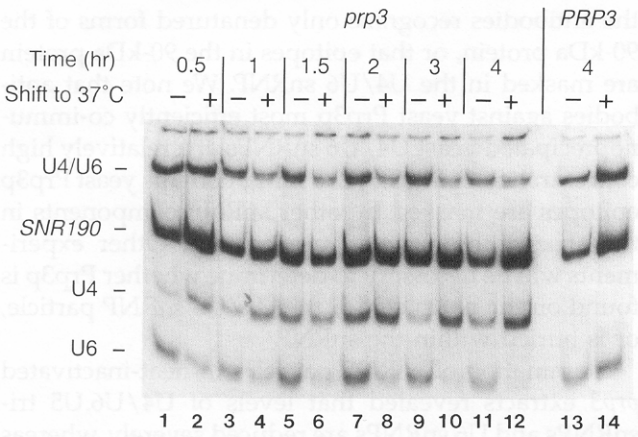

FIGURE 3. Free U4 snRNA accumulates and free U6 snRNA and U4/U6 complexes diminish in a *prp3-1* strain shifted to the nonpermissive temperature. Cells from a *prp3-1* strain JWY3000 or a *PRP3* wild-type strain JWY2846 were grown to mid-log phase at the permissive temperature of 23 °C and either maintained at this temperature or shifted to 37 °C for the indicated length of time. RNA was extracted and annealed to radiolabeled oligonucleotides complementary to the U4, U6, and snR190 snRNAs, under conditions in which the base pairing interactions between the U4 and U6 snRNAs are stable. snRNA/oligomer complexes were then separated on a nondenaturing polyacrylamide gel. Note the presence of free U4 snRNA in the *prp3-1* strain at both the permissive and nonpermissive temperatures.

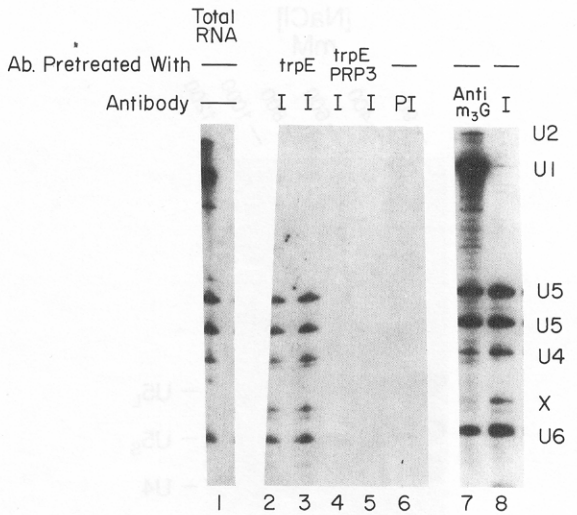

FIGURE 4. Co-immunoprecipitation of U4, U5, and U6 snRNAs from yeast splicing extracts by anti-Prp3p antibodies. Splicing extracts prepared from wild-type yeast BJ2407 were incubated with anti-Prp3p IgG bound to protein A–Sepharose. Immune complexes were collected by centrifugation, and RNA was extracted, resolved by polyacrylamide/urea gel electrophoresis, electroblotted to Nytran, and probed with ^{32}P-labeled cloned DNA encoding U4, U5, or U6 snRNAs. The positions of U4, U5L, U5S, and U6 snRNAs and band X are shown. Lane 1, total RNA from splicing extract; lanes 2 and 3, RNA precipitated with anti-Prp3p antibodies that had been pre-incubated with an *E. coli* extract containing TrpE protein; lanes 4 and 5, RNA precipitated with anti-Prp3p antibodies that had been neutralized by pre-incubation with an *E. coli* extract containing TrpE-Prp3p fusion protein; lane 6, result of incubating extract treated with (Prp3p) pre-immune serum; lane 7, RNA precipitated with anti-trimethylguanosine antiserum; lane 8, RNA precipitated by anti-Prp3p antibodies with no preincubation.

(600 mM and 800 mM) reduced the precipitation of both U5L and U5S greatly, with less effect on U4 or U6 (Fig. 5). None of these snRNAs was precipitated in the presence of 1,000 or 1,200 mM NaCl. Thus, Prp3p is associated more specifically and tightly with the U4/U6 snRNP and is present in the U4/U6.U5 tri-snRNP.

We also detected an snRNA, labeled band X, that is significantly enriched in anti-Prp3p immunoprecipitates of splicing extracts, including those formed at up to 800 mM Na^+ plus K^+ (Fig. 5). Hybridization of various synthetic oligonucleotides complementary to sequences within U4 snRNA revealed that band X RNA is derived from U4 snRNA by degradation or cleavage near the 3′ end of U4 (data not shown). We conclude that nt 136–157 at the 3′ end of U4 are not necessary for co-immunoprecipitation of U4 snRNA by anti-Prp3p.

Prp3p is homologous to a human U4/U6 snRNP protein

The nucleotide sequence of *PRP3* contains an uninterrupted 1,407-bp open reading frame encoding a 469-amino acid polypeptide with a calculated molecular weight of 55,876 Da. Recently, a cDNA encoding the human homologue of Prp3p, Hprp3p, has been isolated (Genbank accession no. AA205409; Lauber et al., 1997; J. Hu, pers. comm.). Hprp3p is a predicted 77-kDa protein that migrates on SDS gels as a 90-kDa protein. Hprp3p is a component of the U4/U6 snRNP, corresponding to the 90-kDa U4/U6 snRNP protein identified previously by Gozani et al. (1994). Alignment of the Prp3p amino acid sequence with that of Hprp3p shows that the two proteins are 27% identical and 55% similar in amino acid sequence. Most of the similarity lies in the C-terminal half of Hprp3p (Lauber et al., 1997 and data not shown). As noted by Lauber et al. (1997), there are also *Caenorhabditis elegans* and *Schizosaccharomyces pombe* homologues of Prp3p (accession nos. Z49128 and Z66525, respectively).

DISCUSSION

In this paper, we have shown that inactivation of Prp3p blocks formation of spliceosomes from prespliceosomes as a result of loss of the U4/U6.U5 tri-snRNPs. Similar results were found upon inactivation or depletion of Prp4p, Prp6p, or Prp8p (Banroques & Abelson, 1989; Abovich et al., 1990; Brown & Beggs, 1992). Like Prp3p, each of these proteins is associated with the U4/U6.U5 tri-snRNP. Both Prp3p and Prp4p appear to be tightly associated with the U4/U6 snRNP and present in the U4/U6.U5 tri-snRNP (Banroques & Abelson, 1989; Peterson-Björn et al., 1989; Bordonné et al., 1990; Xu et al., 1990). Antibodies against either protein co-immunoprecipitate U4, U5, and U6 snRNAs in low

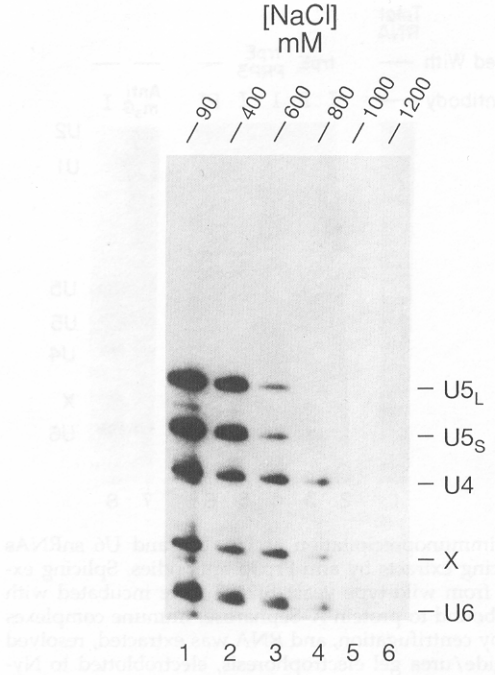

FIGURE 5. Effects of varying NaCl concentration on the immunoprecipitation of U4, U5, and U6 snRNAs with anti-Prp3p antibodies. Splicing extracts from wild-type yeast BJ2407 were adjusted to increasing concentrations of NaC1 (lanes 1–6) prior to the addition of anti-Prp3p antibodies. Immunoprecipitates were washed and RNA was extracted and assayed as described in Materials and Methods. Lane 1, 90 mM NaC1; lane 2, 400 mM NaC1; lane 3, 600 mM NaC1; lane 4, 800 mM NaC1; lane 5, 1 M NaC1; lane 6, 1.2 M NaC1. Positions of U4, U5L, U5S, and U6 snRNAs as well as band X are indicated.

salt (150–400 mM NaCl). U5 snRNA is not precipitated efficiently at higher salt, but U4 and U6 snRNAs are co-immunoprecipitated at salt concentrations up to 750–800 mM. The loss of U5 precipitation at higher salt presumably reflects disruption of the U4/U6.U5 tri-snRNP to U4/U6 and U5 snRNPs (Cheng & Abelson, 1987; Banroques & Abelson, 1989; Abovich et al., 1990; Behrens & Lührmann, 1991). By similar criteria, Prp8p was shown to be associated specifically with the U5 snRNP and present in the U4/U6.U5 tri-snRNP (Lossky et al., 1987), and Prp6p was found to be a U4/U6.U5 tri-snRNP protein that associates via U4/U6 (Abovich et al., 1990; Galisson & Legrain, 1993).

By similar criteria, Lauber et al. (1997) showed that the human homologues of Prp3p and Prp4p are present in the U4/U6.U5 tri-snRNP and are associated specifically with the U4/U6 snRNP. The U4/U6 snRNP containing the human 60-kDa and 90-kDa proteins could be purified by immunoaffinity chromatography with antibodies versus the 60-kDa protein, using buffer containing up to 300–400 mM KCl. None of the other U4/U6.U5-specific proteins copurified under these high-salt conditions. Although anti-60-kDa antibodies could recognize U4/U6 and U4/U6.U5 snRNPs, anti-90-kDa antibodies failed to co-immunoprecipitate snRNPs. This result led Lauber et al. to suggest that the antibodies recognize only denatured forms of the 90-kDa protein, or that epitopes in the 90-kDa protein are masked in the U4/U6 snRNP. We note that antibodies against yeast Prp3p most efficiently co-immunoprecipated yeast U4/U6 snRNPs at a relatively high concentration of $MgCl_2$ (12.5 mM); perhaps yeast Prp3p epitopes are masked by other snRNP components in the absence of elevated magnesium. Other experiments will be necessary to determine whether Prp3p is found on the periphery of the U4/U6 snRNP particle, or is buried within the snRNP.

Examination of snRNP particles in heat-inactivated *prp3* extracts revealed that levels of U4/U6.U5 tri-snRNPs and U6 snRNPs are reduced severely, whereas levels of U4/U6 snRNPs and U5 snRNPs are not changed significantly. Free U4 snRNPs, which are not found in extracts of wild-type yeast, are present in the *prp3* mutant extract. Examination of in vivo levels of free U4, free U6, and U4/U6 hybrid snRNAs in the *prp3* mutant yielded similar results, but also suggested that some fraction of the U4/U6 snRNP is destabilized. Taken together, these results suggest that Prp3p may be required for formation of stable U4/U6 snRNPs and assembly of U4/U6.U5 tri-snRNPs from U4/U6 and U5 snRNPs. However, our results do not distinguish whether the remaining U4/U6 snRNPs detected on glycerol gradients of *prp3* mutant extracts are stable free U4/U6 snRNPs or are derived from disassembly of the U4/U6.U5 tri-snRNPs, or both.

Analyses of levels of snRNP particles in the *prp4*, *prp6*, and *prp8* mutants have yielded results that partially, but not completely, overlap with those demonstrated here for *prp3* mutants; to some extent, the differences may reflect subtleties in the experimental methods used, as indicated in the previous paragraph for heat-inactivation of *prp3* mutants in vivo versus in vitro heat treatment of *prp3* extracts. In *prp4* mutant strains that have been shifted to the nonpermissive temperature, snRNP levels resemble those seen in *prp3* mutant strains heat-inactivated in vivo. In *prp6* mutant strains shifted to 37 °C, levels of U6 snRNPs are diminished, as seen in heated *prp3* mutants; but levels of U4/U6 snRNPs increase in the *prp6* mutants at the nonpermissive temperature (Galisson & Legrain, 1993). Whether these differences reflect unique functions of the U4/U6 snRNP-associated proteins Prp3p, Prp4p, and Prp6p remains to be established. Effects of depleting the U5 snRNP protein Prp8p are more clear; aberrant U5 snRNPs are formed, presumably causing disassembly of the U4/U6.U5 tri-snRNP (Brown & Beggs, 1992).

The accumulation of the novel U4 snRNP and depletion of the U6 snRNP in heat-inactivated *prp3* extracts may reflect any of a variety of effects on the complex and poorly understood pathways of synthesis, assembly, and recycling of the U4 and U6 snRNP particles. Assembly of U4/U6 snRNPs may be slowed

or blocked in the *prp3* mutant, resulting in formation of an aberrant U4 snRNP; unassembled or aberrantly assembled U6 snRNA might be degraded. Previously assembled U4/U6 snRNPs, including those present in the U4/U6.U5 tri-snRNP, might fall apart upon heat inactivation of Prp3p, resulting in buildup of free U4 snRNPs and destabilization of U6 snRNA. Alternatively, the increase in free U4 snRNA and appearance of a U4 snRNP in the *prp3* mutant also might result from up-regulation of U4 snRNA synthesis. This effect could occur in response to a block in assembly of U4/U6 snRNPs or destabilization of U4/U6 snRNPs. Levels of U4 snRNA increase in strains containing a temperature-sensitive allele of the U4/U6.U5 tri-snRNP protein Prp31p (Weidenhammer et al., 1997), and in strains containing mutations in the genes encoding U4 or U6 snRNAs (Shannon & Guthrie, 1991; Fortner et al., 1994). A G14C mutation in the U4 snRNA destabilizes U4/U6 interactions; fivefold excess U4 snRNA accumulates in this mutant and apparently sequesters excess free U6 snRNA, resulting in increased U4/U6 snRNP and decreased U6 snRNP (Shannon & Guthrie, 1991). In the *prp3* mutant, however, the U4/U6 hybrid may not be able to form from excess U4 snRNA or, once formed, may be unstable, resulting in accumulation of free U4 snRNP and degradation of U6 snRNA. Our data do not distinguish between these models.

Several different results indicate that the Prp3p and Prp4p proteins interact with each other. Increased expression of *PRP3* suppresses the Ts^- phenotype of the *prp4-1*, *prp4-2*, and *prp4-11* alleles, but not the Ts^- phenotype of 12 other *prp4* missense mutant alleles or the lethal phenotype of a *prp4*-Δ null allele (Last et al., 1987; Hu et al., 1994). The *prp3-1* mutation is synthetically lethal in combination with the *prp4-1* mutation; the *prp3-1 prp4-1* double mutant does not grow at 23°, a temperature that is permissive for either of the Ts^- *prp3* or *prp4* mutants. Neither *prp3* nor *prp4* is lethal in combination with any of 18 other *prp* mutants tested (Ruby et al., 1993; Maddock et al., 1994, 1996). Two-hybrid assays reveal physical interactions between Prp3p and Prp4p in yeast (J. Banroques, pers. comm.). In addition, the human homologues of Prp3p and Prp4p appear to interact. Hprp4p from HeLa extracts binds to Hprp3p on an affinity column, and antibodies against Hprp3p co-immunoprecipitate Hprp4p (J. Hu, pers. comm.). The yeast and human Prp4 proteins contain five so-called WD repeats, similar to those in the β-subunit of transducin and other G proteins (Dalrymple et al., 1989). Such repeats are thought to be involved in protein–protein interactions. The missense mutations in *prp4* that are suppressed specifically by overproduction of yeast Prp3p lie within these β-transducin repeats (Hu et al., 1994), suggesting that Prp3p might interact with Prp4p via the WD repeats. Missense mutations in the WD repeats of Prp4p block activation of assembled spliceosomes, suggesting that Prp4p (and perhaps Prp3p) may be involved in conformational rearrangements of spliceosomal components (Ayadi et al., 1997).

Previously, it was found that the 5′ stem loop of U4 snRNA is necessary for association of Prp4p with the U4/U6 snRNP and for association of U4/U6 snRNP with the U5 snRNP. This result led to the suggestion that Prp4p promotes interactions between the U4/U6 snRNP and the U5 snRNP to form the U4/U6.U5 tri-snRNP (Bordonné et al., 1990; Xu et al., 1990). We observed that cleavage of the 5′ 15 nt from U4 snRNA prevented co-immunoprecipitation of U4, U5, and U6 snRNAs by anti-Prp3p antibodies, whereas cleavage of nt 48–65 in stem I of U6 snRNA did not prevent co-immunoprecipitation of these snRNAs (data not shown). Similar results were obtained with antibodies versus Prp4p (Bordonné et al., 1990; Xu et al., 1990). Thus, we conclude that the 5′ end of U4 snRNA is necessary for association of Prp3p with the U4/U6 snRNP, perhaps via direct interactions with the snRNAs, or else via association with Prp4p. Prp3p contains sequences homologues to a ds RNA binding motif in *Escherichia coli* RNase III (Lauber et al., 1997), whereas Prp4p does not contain any known RNA binding motifs. It remains to be tested whether either protein interacts directly with U4 or U6 snRNAs, or with components of the U5 snRNP to promote assembly of the U4/U6 snRNP with the U5 snRNP to form the U4/U6.U5 tri-snRNP.

The identification of a human homologue of Prp3p further exemplifies the conservation of yeast and human splicing factors. More than half of the 40-plus yeast splicing factors thus far identified have human homologues (reviewed in Krämer, 1996; see also Horowitz & Krainer, 1997; Neubauer et al., 1997). Yeast Prp3p and Prp4p and their human homologues are the only U4/U6 snRNP-specific proteins identified thus far. Evidence to date suggests that Hprp3p and Hprp4p, like yeast Prp3p and Prp4p, interact with each other. Regulation of this interaction between Prp3p and Prp4p, perhaps via the WD repeats, may play important roles in the assembly and function of the U4/U6 snRNP in yeast and metazoans.

MATERIALS AND METHODS

Preparation of splicing extracts and substrates

Splicing extracts were prepared from yeast as described in Lin et al. (1985) using yeast strains JWY2878 (*MATa leu2 ura3-52 trp1 prb1-1122 pep4-3 prc1-407 gal2*) and JWY630 (*MATa prp3-1 ade1 ade2 ura1 tyr1 his7 lys2 gal1*). ^{32}P-Labeled intron-containing *ACT1* precursor RNAs were synthesized in vitro using SP6 polymerase according to Lin et al. (1985) and gel-purified prior to use. The specificity of heat inactivation of *prp3* mutant extracts was demonstrated by successful in vitro complementation with other different heat-inactivated *prp* mutant extracts.

Nondenaturing gel electrophoresis of splicing complexes

Vertical polyacrylamide gels for visualization of prespliceosomes and spliceosomes were performed as described in Arenas and Abelson (1993) and Weidenhammer et al. (1997). Depletion of ATP was accomplished by addition of 10 mM glucose (in place of 2 mM ATP) to the reaction mix; samples were incubated at room temperature for 30 min to allow depletion of endogenous ATP.

Glycerol gradient fractionation of snRNP complexes

U6, U4/U6, U5, and U4/U6.U5 snRNP complexes were analyzed by glycerol gradient centrifugation of extracts as described in Weidenhammer et al. (1997). RNA was extracted from fractions collected from each gradient by addition of 35 μg/mL glycogen and 0.4 mL phenol/chloroform/isoamyl alcohol (50:49:1), and was precipitated at −20 °C by addition of 1/10 volume 3 M NaAc, pH 5.5, and 2.5 volume cold EtOH. snRNA species were separated by electrophoresis on 7% polyacrylamide (30:0.8)/4 M urea denaturing gels, electroblotted to Nytran Plus membrane, and detected by hybridization with radiolabeled oligonucleotides U4D (5′-AGGTA TTCCAAAAATTCCCTAC-3′ [nt 158–137]), U5 ew-rd (5′-CTATGGAGACAACACCGG-3′ [nt 129–110]), and U6 ew-rd (5′-CGGTTCATCCTTATGCAGGGG-3′ [nt 86–66]) complementary to the U4, U5, and U6 snRNAs, as described in Bordonné et al. (1990).

Gel electrophoresis of U4 and U6 snRNAs and U4/U6 snRNA hybrids

The relative amounts of free U4 snRNA, free U6 snRNA, and U4/U6 hybrids in vivo were assayed as described in Fortner et al. (1994), using 5 μg RNA per sample from the wild-type yeast strain JWY2846 (*MATα ura3-52 Δtrp1 leu2-3,112 his3-11,15 lys2*) and the otherwise isogenic *prp3-1 URA3* strain JWY3000 (*MATα ura3-52 Δtrp1 leu2-3,112 his3-11,15 lys2 PRP3::prp3-1 YIp5*). Strains were grown at 23 °C to ~0.5 × 10^8 cells/mL; for temperature shift experiments, the cultures were then diluted with an equal volume of fresh medium and maintained at 23 °C or diluted with an equal volume of fresh medium at 51 °C and shifted to 37 °C for the indicated time. Cells from a 10-mL culture were harvested by centrifugation at 3,000 × *g* for 5 min, washed once with 0.5 volume RE buffer (100 mM LiCl, 100 mM Tris-HC1, pH 7.5, 1 mM EDTA), and suspended in 0.4 mL RE buffer. This solution was transferred to a fresh tube containing ~2/3 volume glass beads and vortexed 4 min at 4 °C. Proteins were removed by sequential extractions with 0.3 mL equilibrated phenol (United States Biochemical, Cleveland, Ohio), 0.3 mL phenol/chloroform/isoamyl alcohol (50:49:1), and 0.3 mL chloroform. RNA was precipitated at −20 °C overnight and suspended in 0.1 mL DEPC-treated dH_2O; RNA was stored at −80 °C. Oligonucleotides U4D and U6D (5′-AAAACGAAATAAATCTCTTTG-3′ [nt 112–92]) complementary to U4 and U6 snRNAs, respectively, and oligonucleotide 190, complementary to snoRNA snR190 (5′-GGCTCAGATC TGCATGTGTTGTATAACACTGG-3′ [nt 158–190]), were 5′ end-labeled with ^{32}P using T4 polynucleotide kinase and used as probes. The following components were combined in an Eppendorf tube on ice in the order given: 0.5 μL 1 M Tris, pH 7.5, 0.5 μL 20 mM EDTA, pH 8.0, 0.5 μL 3 M NaCl, and DEPC-treated dH_2O such that the final volume, after addition of RNA, would equal 4 μL. Five micrograms RNA were then added; for control reactions, the sample was incubated at 70 °C for 3 min. Samples were subjected to gel electrophoresis as described in Weidenhammer et al. (1997).

Antibodies against Prp3p

Both TrpE fusion proteins and synthetic peptides conjugated to keyhole limpet hemocyanin (KLH) were used as immunogens. A 1,371-bp *Eco*R I–*Hin*d III fragment containing codons 79–469 of the *PRP3* open reading frame was used to construct a *TrpE-PRP3* fusion in plasmid pATH11 (Dieckman & Tzagoloff, 1985). Production of TrpE-Prp3p fusion protein in *E. coli* was induced by the addition of 20 μg/mL 3β indoleacrylic acid for 4 h. After induction, cells were harvested by centrifugation at 4,000 × *g* for 10 min and suspended in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 3 mg/mL lysozyme) for 2 h. NaCl was added to 300 mM, and NP-40 to 0.6% (vol/vol). The suspension was incubated on ice for 30 min, sonicated, and washed by centrifugation at 12,000 × *g* for 10 min in 1 M NaCl, 10 mM Tris, pH 7.5, and twice in 10 mM Tris, pH 7.5. The resulting pellet, highly enriched in fusion protein, was used in affinity blocking experiments (see below). Fusion proteins were purified further by electrophoresis in SDS-polyacrylamide gels (Laemmli, 1970) and electroelution from gel slices in 50 mM NH_4HCO_3, 0.05% SDS. Synthetic peptides corresponding to amino acids 1–14 of Prp3p plus a cysteine [sequence = (C)MPPRNTYEKGN PKR] were purchased from Biosearch Research Chemicals (San Rafael, California), and conjugated to KLH as described in Moehle et al. (1989).

Rabbits were injected subcutaneously with 200 μg of fusion protein or peptide (coupled to KLH) in Freund's complete adjuvant, and boosted subsequently by monthly intramuscular injections with 100 μg of fusion protein or peptide conjugate in Freund's incomplete adjuvant. Rabbits were immunized with TrpE-Prp3p under contract with Berkeley Antibody Company, Inc. (Richmond, California). Resulting antisera detected both the TrpE-Prp3p fusion protein containing amino acids 79–469 of Prp3p and an OmpF-Prp3p-β-galactosidase trihybrid protein (Last & Woolford, 1986) containing amino acids 330–432 of Prp3p (data not shown). The antisera recognized a 56-kDa protein on immunoblots of whole-cell extracts of yeast overproducing *PRP3* mRNA, and immunoprecipitated a radioactively labeled 56-kDa protein synthesized in vitro from synthetic *PRP3* mRNA (data not shown). Pre-immune sera gave no signal in any of these assays with Prp3p antigens. We conclude that these antisera bind to and precipitate Prp3p identical in size to that predicted from the *PRP3* nucleotide sequence.

Immunoprecipitation of snRNAs from splicing extracts

Splicing extracts (10 μL) from wild-type yeast strain BJ2407 (*MATa/MATα leu2/leu2 trp1/trp1 ura3-52/ura3-52 prb1-1122/prb1-1122 prc1-407/prc1-407 pep4-3/pep4-3*) were mixed with an equal volume of splicing buffer (final concentrations

60 mM KPO_4, pH 7.0, 3% wt/vol PEG, 2.5 mM $MgCl_2$, 2 mM ATP), incubated 25 min at 23 °C, and then placed on ice. For precipitation with the anti-Prp3p antibody, 12.5 mM $MgCl_2$ was found to be optimal. All samples were adjusted to 30 mM KCl, 120 mM NaCl, 0.1% (vol/vol) NP-40, 10 mM HEPES, pH 7.9, 1.5% (wt/vol) PEG 6000, 4 mM EDTA, and incubated with protein A–Sepharose bound IgG for 2 h at 4 °C with mixing. The samples were then centrifuged at 14,000 × *g* for 1 min, and the pellets washed four times with 500 μL NTN buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% NP-40) and once with 500 μL NT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5). The immunoprecipitates were treated with proteinase K, RNA was extracted with phenol:chloroform (1:1) and precipitated with ethanol. The RNA samples were dissolved in 70% formamide and resolved by electrophoresis on 6% polyacrylamide/7 M urea gels. The gels were electroblotted to Nytran (Schleicher and Schuell) in 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0, for 1 h at 65 V and the filters irradiated for 5 min with ultraviolet light using a transilluminator. The filters were hybridized with ^{32}P-labeled cloned genes or oligonucleotides complementary to yeast snRNAs U1, U2, U4, U5, and U6, washed, and exposed to X-ray film. Relative amounts of each snRNA were quantified from the X-ray films, using a Zeineh Soft Laser Scanning Densitometer.

ATP or $MgCl_2$ concentrations were varied only in the first step during incubation of extracts with splicing buffer. Monovalent ion (Na^+/K^+) concentration was varied only during the second step prior to addition of antibody bound to protein A–Sepharose. In all cases, the concentration of KCl was 30 mM, whereas NaCl concentrations were varied.

DNA sequencing

The 1.7-kb *Bam*H I/*Nde* I fragment containing *PRP3* (Last et al., 1984) was subcloned in opposite orientations into M13mp18 or M13mp19 (Norrander et al., 1983). A series of 5′ or 3′ deletions of *PRP3* was created from these DNAs (Dale et al., 1985), and used as templates for DNA sequencing by the dideoxy chain-termination method (Sanger et al., 1977).

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