1164–1170 *Nucleic Acids Research, 1996, Vol. 24, No. 6*

The *PRP31* gene encodes a novel protein required for pre-mRNA splicing in *Saccharomyces cerevisiae*

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Received July 31, 1995; Revised and Accepted January 19, 1996

GenBank accession no. U31970

ABSTRACT

The pre-mRNA splicing factor Prp31p was identified in a screen of temperature-sensitive yeast strains for those exhibiting a splicing defect upon shift to the non-permissive temperature. The wild-type *PRP31* gene was cloned and shown to be essential for cell viability. The *PRP31* gene is predicted to encode a 60 kDa polypeptide. No similarities with other known splicing factors or motifs indicative of protein–protein or RNA–protein interaction domains are discernible in the predicted amino acid sequence. A *PRP31* allele bearing a triple repeat of the hemagglutinin epitope has been generated. The tagged protein is functional *in vivo* and a single polypeptide species of the predicted size was detected by Western analysis with proteins from yeast cell extracts. Functional Prp31p is required for the processing of pre-mRNA species both *in vivo* and *in vitro*, indicating that the protein is directly involved in the splicing pathway.

INTRODUCTION

The removal of intervening sequences from precursor messenger RNAs (pre-mRNAs) is a critical processing step in the maturation of transcripts. The catalytic reactions of splicing require association of the substrate molecule with numerous *trans*-acting factors to form the spliceosome (for reviews see 1–4 and references therein). Five small nuclear RNAs (snRNAs), associated into ribonucleoprotein particles (snRNPs), assemble into a splicing complex with the pre-mRNA. The U1 snRNA base pairs with the 5′ splice site (5–7), resulting in formation of the commitment complex (8–10). The U2 snRNP then interacts with the branch point sequence in an ATP-dependent fashion to form the pre-spliceosome (11–15). The U4/U6 and U5 snRNPs associate with this complex as a single, tri-snRNP particle to form the spliceosome (11,16–18). Conformational rearrangements then occur, allowing cleavage at the 5′ splice site and, subsequently, cleavage at the 3′ splice site and ligation of the two exons to form the mature mRNA species.

Current models predict that splicing is catalyzed by the snRNAs and experimental evidence from both yeast and mammals supports this hypothesis (19–23). However, the splicing machinery requires a large number of protein factors to accurately function *in vivo*. More than 30 gene products have been identified as essential for the splicing reaction in *Saccharomyces cerevisiae* (for reviews see 2,24 and references therein). A number of these factors have been identified in screens of temperature-sensitive strains for those exhibiting a splicing defect at the non-permissive temperature. These are referred to as *PRP* genes, for precursor RNA processing (25–29). Isolation of suppressors of mutant alleles of the *PRP* genes or of mutated splice sites in pre-mRNAs has revealed a number of novel gene products required for splicing to occur (30–32). Still other factors have been identified by assaying for mutations in genes that in combination with a mutation in one of the snRNAs confer synthetic lethality (33,34).

In order to identify novel *trans*-acting proteins required for pre-mRNA splicing in *S.cerevisiae*, we isolated temperature-sensitive mutant strains that accumulate pre-mRNA *in vivo* after shift to the non-permissive temperature of 37°C. Six new genes were identified and were designated *PRP29–34* (35). In this report we describe the isolation and characterization of the *PRP31* gene. The 60 kDa gene product is essential for vegetative growth in *S.cerevisiae*. Furthermore, we demonstrate specific inactivation of the *in vitro* splicing activity of extracts derived from a *prp31-1* strain, consistent with a direct requirement for Prp31p in processing of pre-mRNA species. Interactions with a variety of known splicing factors were examined by genetic and biochemical analyses.

MATERIALS AND METHODS

Strains and media

Strains used in this study are listed in Table 1. Standard media and techniques were utilized for growth and manipulation of yeast and bacteria (36). Transformations were performed by the LiAc method of Ito *et al.* (37).

Cloning and subcloning of *PRP31*

Yeast strain JWY771 was transformed with a library of *Sau*IIIA-digested fragments of yeast genomic DNA cloned in YCp50 (38). Cells were plated on selective medium and placed at 23°C for 18–20 h. Seventy five percent of the plates were then shifted to 37°C to select for Ts^+ transformants. The remaining plates were kept at 23°C to estimate the total numbers of transformants examined; these transformants were then picked, patched and replica-plated to check for growth at 37°C. Fragments to be assayed for complementing activity were subcloned into pRS316 (39). For high copy suppression analysis the 3.0 kb *Hin*dIII fragment was subcloned into pRS426 (41).

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Table 1. Strains used

Strain	Genotype	Source
JWY771	MAT**a** *prp31-1 his4-519 ura3-52 lys2 cry2*::*LEU2*	65
DBY1034	MAT**a** *his4-519 ura3-52 lys2-801*	D. Botstein
JWY2861	MAT**a** *prp31-1 trp1 ura3-52*	This study
LP112	MAT**a**/MATα *can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3, 112 trp1-1/trp1-1 ura3-1/ura3-1 ade2-1/ade2-1*	J. Friesen
JWY2964	*prp31*::*TRP1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* [PRP31/pRS316]	This study
JWY2857	MAT**a** *prp31-1 ade1 leu2-3,112 trp1 ura3-52*	This study
JWY657	MAT**a** *prp2-1 ura3-52 ade2 His*$^-$	65
JWY630	MAT**a** *prp3-1 ade1,2 ura1 tyr1 his7 lys2 gal1*	J. Abelson
JWY688	MAT**a** *prp6-1 leu2-3,112 lys2 tyr1*	65
JWY715	MAT**a** *prp8-1 leu2-3,112 his7 Lys*$^-$	65
JWY775	MAT**a** *prp16 his4 ura3-52 lys2 Δtrp1*	65
JWY803	MAT**a** *prp22-1 ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
JWY806	MAT**a** *prp24-1 ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
BJ2168	MAT**a** *leu2 ura3-52 trp1 prb1-1122 pep4-3 prc1-407 gal2*	E. Jones
JWY675	MAT**a** *prp3-1 ura3-52 ade1*	65
JWY701	MAT**a** *prp4-1 ura3-52 his7 ade1*	65
JWY732	MAT**a** *prp5-1 ura3-52 lys2 his7*	65
JWY784	MAT**a** *prp6 his4-519 ura3-52 lys2 cry2*::*LEU2*	65
JWY666	MAT**a** *prp8-1 ura3-52 ade2 his7*	65
JWY664	MAT**a** *prp9-1 ura3-52 ade2 His*$^-$	65
JWY623	MAT**a** *prp11-1 ura3-52 leu2-3,112 trp1 lys2 gal1 tyr1*	65
JWY792	MAT**a** *prp17 ade2-101 his3Δ200 ura3-52 lys2-801*	J. Abelson
JWY794	MAT**a** *prp18 ade2-101 his3Δ200 ura3-52 lys2-801*	65
JWY796	MAT**a** *prp19 ade2-1 his3Δ200 ura3-52 lys2-801 leu2*	65
JWY798	MAT**a** *prp20 ade2-101 his3Δ200 ura3-52 tyr1*	65
JWY800	MAT**a** *prp21 ade2-101 his3Δ200 ura3-52 tyr1*	65
JWY802	MAT**a** prp22 ade2-101 his3Δ200 ura3-52 tyr1	65
JWY804	MAT**a** *prp23 ade2-101 his3Δ200 ura3-52 tyr1 lys2-801*	65
JWY806	MAT**a** *prp24 ade2-101 his3Δ200 ura3-52 lys2-801*	65
JWY808	MAT**a** *prp25 ade2-101 his3Δ200 ura3-52 tyr1*	65
JWY810	MAT**a** *prp26 ade2-101 his3Δ200 ura3-52 tyr1*	65
JWY812	MATα *prp27 ade2-101 his3Δ200 ura3-52 lys2-801*	65
JWY2871	MAT**a** *prp31-1 his3 leu2-3,112 lys2 ura3-52*	This study
JWY2885	MAT**a** *prp32 leu2 lys2 ura3-52 His*$^-$	This study
JWY2419	MAT**a** *snr14*^{ts} *lys2-801 ura3-52 leu2-3,112 his4 trp1Δ*	67
JWY2402	MATα *prp18 ura3-52 lys2-801 his3*	67
JWY695	MAT**a** *prp4-1 leu2-3,112 lys2 his7 tyr1 ade2*	65
JWY40	MAT**a** *his3Δ200 leu2Δ1 lys2-801 trp1Δ101 ura3-52 spp2Δ1*::*LEU2 + pM2 (spp2-1)*	32
SC252	MAT**a** *ura3-52 ade1 leu2-3,112*	J. Hopper
JWY2862	MATα *prp31-1his3 his4-519 lys2 ura3-52*	This study
JWY2865	MAT**a** *prp31-1 ade1 leu2-3,112 lys2 ura3-52*	This study
JWY2866	MAT**a** *his4-519 lys2 ura3-52*	This study
JWY2867	MATα *his3 leu2-3,112 ura3-52*	This study
JWY2868	MATα *prp31-1 ade1 his3 his4-519 ura3-52*	This study
hs87 (2b)	MAT**a** *prp34-1 trp1 leu2-3,112 lys2-801 his3*	67
JWY721	MATα *prp11-1 his7 tyr1 ade2 leu2-3,112*	65

RNA isolation and analysis

For RNA isolation, yeast 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 shifted to 37°C for 2 h. Cells from a 10 ml culture were harvested by centrifugation at 3000 *g* for 5 min, washed once with 0.5 vol RE buffer (100 mM LiCl, 100 mM

Tris–HCl, pH 7.5, 1 mM EDTA) and suspended in 0.4 ml RE buffer. This solution was transferred to a fresh tube containing an ~2/3 volume of glass beads and vortexed for 4 min at 4°C. Proteins were removed by sequential extractions with 0.3 ml equilibrated phenol (United States Biochemicals, Cleveland, OH), 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.

Analysis of pre-mRNA accumulation was by electrophoresis of RNA on 1% agarose–6% formaldehyde gels as described by Moritz *et al.* (42). RNA samples were prepared as in Feinberg and Vogelstein (43) except that the sample buffer did not contain glycerol. Thirty to forty micrograms of RNA were loaded for Northern analysis. Gels were dry blotted to Nytran Plus membrane (Schleicher & Schuell, Keene, NH). Hybridization with radiolabeled probes has been described (44,45). A 2.2 kb *Hin*dIII fragment bearing the *CRY1* and *SNR189* genes was utilized as a probe.

Transcript and chromosome mapping

Radiolabeled transcripts were generated using the T7 and T3 promoters (Stratagene, La Jolla, CA) flanking the *Bgl*II–*Sac*I fragment of *PRP31* in pRS316. These probes were hybridized to a blot of poly(A)$^+$ RNA. Labeling, hybridization and wash conditions were performed as instructed by the manufacturer.

A blot of chromosomal DNA separated by CHEF was obtained from Clontech Laboratories Inc. (Palo Alto, CA) and hybridized with the 3.0 kb *Hin*dIII fragment bearing the *PRP31* gene. Hybridization and wash conditions were performed as per Clontech's instructions.

DNA sequence determination and analysis

The *PRP31* gene was sequenced by the dideoxy chain termination method of Sanger (46), using Sequenase enzyme (United States Biochemicals, Cleveland, OH) as per the manufacturer's instructions. Restriction fragments for sequencing were subcloned into Bluescript pKS$^+$ and pSK$^+$ vectors (Stratagene). Synthetic oligonucleotides used as primers were synthesized by Operon Technologies (Alameda, CA). Sequence analysis and homology searches were carried out using the Genetics Computer Group's Sequence Analysis Software Package (access to this program was courtesy of the Pittsburgh Supercomputing Center).

Construction of a *prp31*::*TRP1* null allele

A 0.9 kb *Bgl*II–*Sac*I fragment containing the *S.cerevisiae TRP1* gene (47) was subcloned into the 3.0 kb *Hin*dIII fragment containing *PRP31* in pRS316 modified such that the *Sac*I and *Eco*RI sites within the polylinker were destroyed. This construct was digested with *Sal*I to release a 1.4 kb fragment (Fig. 2) and transformed into yeast diploid strain LP112. Sporulation and dissection of transformants yielded only two viable Trp$^-$ spore clones. The disruption event was confirmed by Southern analysis of genomic DNA. To confirm that spore inviability was due to disruption of the *PRP31* gene, a *PRP31*/*prp31*::*TRP1* diploid was transformed with either the 3.0 kb *Hin*dIII fragment or the 1.4 kb *Eco*RI–*Sac*I fragment bearing the *PRP31* gene in pRS316. Transformants were then sporulated and tetrads dissected. All Trp$^+$ spore clones were also Ura$^+$ and 5FOA^s, indicating that the plasmid-borne gene is essential for vegetative growth of these strains.

Epitope tagging and detection of Prp31p

A *Bgl*II site was introduced downstream of the *Eco*RI site in *PRP31* by oligonucleotide-directed mutagenesis of the 0.6 kb *Hin*dIII–*Eco*RI fragment of *PRP31* in pRS316. A *Bgl*II fragment bearing three tandem repeats of the hemagglutinin epitope (courtesy of Dr Bruce Futcher) was subcloned into the engineered site. Introduction and orientation of the epitope was confirmed by restriction enzyme analysis and sequencing of the construct. The remainder of the *PRP31* gene was then introduced into the tagged fragment by subcloning.

Protein extraction for Western analysis was as described previously (42). Samples were loaded onto 3% polyacrylamide (30:0.44) stacking–10% polyacrylamide (30:0.8) running gels. After electrophoresis proteins were transferred to nitrocellulose. Transfer efficiency and marker protein migration were determined by staining with Ponceau S solution (Sigma, St Louis, MO). The blot was then destained with dH_2O and probed with anti-HA monoclonal antibodies as in Deshmukh *et al.* (48). Immunoblots were developed with horseradish peroxidase-conjugated anti-rabbit antibodies using an enzyme chemiluminescence kit (Amersham, Arlington Heights, IL) as per the manufacturer's instructions.

In vitro splicing

Cell extracts were prepared according to Lin *et al.* (49). Radiolabeled substrate was produced by *in vitro* transcription of a synthetic actin substrate from the SP6 promoter, using [^{32}P]UTP at 2 mCi/ml reaction. Splicing assays were conducted at 18°C for 1 h, as described in Lin *et al.* (49). Micrococcal nuclease digestion of snRNAs was performed as in Tarn *et al.* (50). Splicing intermediates and products were separated by denaturing gel electrophoresis on 6% polyacrylamide (29:1)–8 M urea gels. For biochemical complementation assays equal volumes of two heat-inactivated *prp* extracts or heat-inactivated *prp* extract and micrococcal nuclease-treated wild-type extract were combined prior to substrate addition. Extracts were heat inactivated as follows: *prp2-1*, JWY657, 37°C, 30 min; *prp31-1*, JWY2857, 37°C, 30 min; U4^{ts}, JWY2419, 42°C, 15 min; *prp24-1*, JWY806, 37°C, 40 min; *prp3-1*, JWY690, 37°C, 40 min.

RESULTS AND DISCUSSION

Cloning of the *PRP31* gene

Strains bearing the *prp31-1* mutant allele are temperature sensitive for growth. In addition, pre-mRNA processing is blocked after a 2 h shift to the non-permissive temperature of 37°C (35). RNA was extracted from cells grown to mid-log phase at the permissive temperature of 23°C and then either maintained at 23°C or shifted to 37°C and subjected to Northern analysis using radiolabeled fragments of the intron-containing *CRY1* and *ACT1* genes as probes. The amount of pre-mRNA was increased and the levels of mRNA were decreased in the strain initially designated hs29 after a shift to the non-permissive temperature (Fig. 1, lanes 3 and 4). Subsequent experiments demonstrated that pre-mRNA accumulates in this strain within 30 min after shift (data not shown).

Genetic analyses indicated that the temperature sensitivity and *in vivo* splicing defect of hs29 co-segregated in crosses to wild-type yeast (Fig. 1, lanes 5–12), indicating that both phenotypes are due to a mutation at a single locus, which we

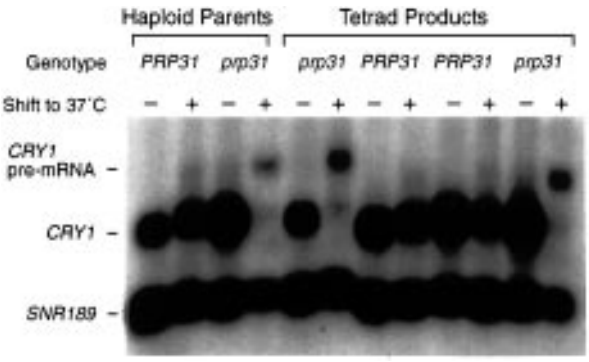

Figure 1. Unspliced pre-mRNA accumulates in temperature-sensitive *prp31* strains shifted to 37°C. Northern analyses of RNA isolated from the haploid parents (SC252, JWY2862; lanes 1–4) and the four spores of an out-crossed tetrad (JWY2865–2868; lanes 5–12) are shown. Cultures were maintained at 23°C or shifted to 37°C for 2 h prior to RNA extraction. A radiolabeled fragment of DNA bearing the *CRY1* and *SNR189* genes was utilized as a probe. Temperature-sensitive strains are indicated as those bearing the *prp31* mutant allele. *CRY1* pre-mRNA and mature RNA species are indicated; *SNR189* serves as a loading control.

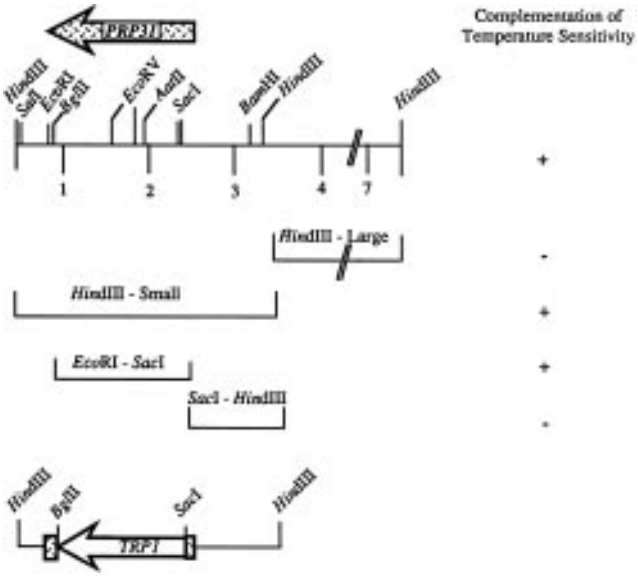

Figure 2. Restriction enzyme map and subcloning analysis of *PRP31*. Fragments were subcloned into pRS316 and transformed into *prp31-1* strain JWY2861 to test for complementation activity. +, ability of the transformants to grow at 37°C; – failure to grow at elevated temperatures. The direction of transcription is indicated by the arrow at the top. Shown at the bottom is the *prp31*::*TRP1* deletion–insertion construct.

designate *PRP31*. The *PRP31* gene was cloned by complementation of the temperature-sensitive defect. A YCp50-based library of yeast genomic DNA was transformed into the *prp31-1 ura3-52* strain JWY771 and transformants that could grow at the previously non-permissive temperature of 37°C were selected for further analysis. Four Ts^+ transformants were obtained from ~60 000 yeast transformants screened. Transformants that had lost the *URA3*-bearing plasmid were no longer able to grow at the non-permissive temperature, indicating plasmid-borne suppression of the growth defect. All complementing plasmids contain identical ~8 kb fragments of yeast genomic DNA. Subcloning analysis defined the complementing activity to a 1.35 kb *Eco*RI–*Sac*I fragment (Fig. 2).

To demonstrate that the complementing DNA corresponds to the *bona fide PRP31* gene, rather than to an extragenic suppressor of the *prp31-1* defect, a 3.0 kb *Hin*dIII fragment from the complementing plasmid was subcloned into the *URA3*-marked integrating vector YIp5 (51). This construct was linearized with *Sac*I and transformed into yeast strain DBY1034 (*PRP31 ura3*-52). Integration of the fragment at the targeted locus was confirmed by Southern analysis of genomic DNA (40) derived from Ura^+ transformants. Integrants were mated to JWY2861 (*prp31-1 ura3-52*) and diploids were sporulated. Of 35 four spore tetrads analyzed all gave rise to two Ts^+Ura^+ spore clones and two Ts^-Ura^- spore clones, indicating that the transformed fragment had integrated within ~1 map unit of the *PRP31* gene.

PRP31 is an essential single copy gene

Hybridization of genomic DNA indicated that the *PRP31* gene is present at single copy in haploid yeast strains, on chromosome XV (data not shown). An ~1.4 kb message was recognized on a blot of poly(A)$^+$ RNA upon hybridization with a radiolabeled probe derived from the *Eco*RI–*Sac*I fragment of the *PRP31* gene. The direction of transcription is indicated in Figure 2.

The *PRP31* gene was determined to be essential by deletion–insertion mutagenesis. The yeast *TRP1* gene was inserted between the *Bgl*II and *Sac*I sites of the *PRP31* gene (Fig. 2). One wild-type allele of the *PRP31* gene was replaced by this fragment in the wild-type diploid strain LP112. Sporulation of this *PRP31*/*prp31*::*TRP1* diploid strain and tetrad dissection yielded only two viable Trp^- spore clones in the 35 tetrads examined. This reduced spore viability could be rescued by introduction of a plasmid-borne copy of the *PRP31* gene prior to sporulation. These data indicate that the *PRP31* gene is required for vegetative growth in *S.cerevisiae.*

Sequence of the *PRP31* gene

Sequence analysis of the complementing DNA revealed a potential open reading frame (ORF) of 1485 bp. Surprisingly, this ORF extends nine codons upstream of the second *Sac*I site and 40 codons downstream of the *Eco*RI site. The *Eco*RI–*Sac*I fragment was able to complement the *prp31*::*TRP1* null allele, as well as the *prp31-1* Ts^- allele (data not shown), indicating that: (i) the C-terminal portion of Prp31p is not required for stability or function of the protein; (ii) some portion of the N-terminal segment of the protein may not be required or translation may initiate from a codon other than the first methionine residue. Primer extension analysis mapped the 5′-ends of the *PRP31* transcripts to nucleotides –60, –65 and –73 5′ of the first ATG of the ORF, indicating that this first ATG is most likely the initiation codon for the wild-type *PRP31* gene (data not shown). Thus complementation by the *Eco*RI–*Sac*I subclone may result from use of a cryptic promoter in the plasmid vector and a different ATG initiation codon. The inferred amino acid sequence of the *PRP31* gene product does not contain any obvious motifs or homologies to sequences currently in the GenBank Database (version 8).

The gene is predicted to encode a product of ~60 kDa. The *PRP31* gene was epitope tagged by insertion of a triple hemagglutinin epitope at the 3′-end of the gene, 32 bp downstream of the *Eco*RI

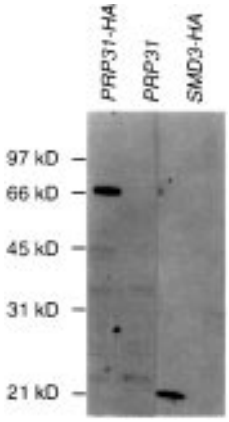

Figure 3. Detection of the HA epitope-tagged allele of *PRP31* by Western blot analysis. Protein extracts were obtained from strains containing the HA-tagged allele of *PRP31* or an untagged allele of the gene. Anti-HA antibodies recognize a single protein species migrating at the predicted size for Prp31–HA in the strain bearing the tagged allele of *PRP31*; no proteins are recognized in the strain bearing the untagged allele of *PRP31*. As a control a strain bearing an HA-tagged allele of the *SMD3* gene (66) was analyzed simultaneously. Migration of molecular weight standards is indicated.

site. To determine whether the tagged allele was functional the construct was subcloned into the *HIS3*-marked plasmid pRS313 and introduced into JWY2964 (*prp31*::*TRP1* [*PRP31*/pRS316]). Transformants that had lost the *URA3*-containing helper plasmid were then selected by plating His^+ transformants on 5-FOA. The Ura^-His^+ colonies obtained demonstrated no temperature sensitivity or other growth defects. The tagged protein could be detected by Western blot analysis (Fig. 3) of cell extracts derived from yeast strains bearing *PRP31-HA* on a CEN-based plasmid. The tagged protein migrates at ~65 kDa, in agreement with the size predicted if translation initiation occurs at the first methionine residue in the ORF, however, post-translational modification of a smaller gene product, initiating from a downstream methionine residue, could yield similar results.

A *prp31-1* strain is defective for splicing *in vitro* as well as *in vivo*

Many strains defective in pre-mRNA processing have been shown to be temperature sensitive for splicing *in vitro* as well as *in vivo*. This result is taken as evidence that the mutations in these strains affect a gene product with a direct role in splicing *per se*, rather than exhibiting an indirect effect due to a defect in transcription, translation or transport of splicing factors (52). In order to determine whether Prp31p is directly required for splicing extracts were prepared from the *prp31-1* strain JWY2857. These extracts are capable of splicing an *in vitro* transcribed synthetic actin substrate, resulting in formation of the lariat intron–3′ exon intermediate and the lariat intron and mature mRNA products (Fig. 4A, lane 1). Upon pre-incubation at increased temperatures prior to substrate addition, the *prp31* extract is no longer able to splice the actin substrate (Fig. 4A, lane 2). Accumulation of pre-mRNA was observed, with no intermediate species being detected, consistent with the *in vivo* splicing defect. Heat inactivation of *prp31* extracts is consistent with a direct involvement of Prp31p in the splicing pathway. Furthermore, since no intermediate species were observed *in vitro* following heat inactivation of the extract or *in vivo* following a temperature shift, the Prp31p protein must play a role prior to the first cleavage reaction. An additional role in subsequent stages of splicing cannot be ruled out at this time.

The *in vitro* temperature sensitivity of a *prp31* extract is due to specific inactivation of a factor required for pre-mRNA splicing

While heat inactivation of extracts derived from a *prp31-1* strain is most likely due to a loss of Prp31p function, the possibility formally exists that the extract is generally deficient for splicing activity following incubation at elevated temperatures. To ascertain that the *in vitro* splicing defect is due to specific inactivation of Prp31p, biochemical complementation assays were performed. Heat-inactivated *prp31* extract was combined with an equal volume of heat-inactivated extract derived from a *prp2-1* strain prior to addition of radiolabeled substrate, splicing was allowed to proceed and the reactions were analyzed for formation of splicing intermediates and products. Prp2p is required for the first cleavage reaction of splicing but is not required for spliceosome assembly (53). Combining the two heat-inactivated extracts restored splicing activity (Fig. 4A, lane 7). Micrococcal nuclease treatment of an extract derived from wild-type strain BJ2168 depletes the extract of snRNAs and rendered this extract unable to splice *in vitro* (Fig. 4A, lane 6). Extract depleted in this way was able to complement heat-inactivated *prp31* extract (Fig. 4A, lane 8). This complementation confirms that the (sn)RNA components of the *prp31* extract are still intact upon heat inactivation. Taken together these results are consistent with a model in which the temperature sensitivity of the *prp31* extract is a result of the specific inactivation of an exchangeable protein factor directly required for pre-mRNA splicing *in vitro* as well as *in vivo*.

Analysis of potential interactions of Prp31p with other splicing factors

Identification of interactions between gene products, either genetically or biochemically, can provide information about the potential functions of splicing factors. To assess potential interactions between Prp31p and other PRP proteins biochemical complementation assays were performed. Extracts made from strains bearing mutations in gene products that are components of the same snRNP particle or are required to interact during splicing may be unable to complement each other in *in vitro* biochemical complementation assays. These experiments thus provide a mechanism for identifying potential interactions between components of the splicing machinery. Heat-inactivated *prp31* extracts were combined with equal volumes of heat-inactivated extracts derived from different *prp* strains, splicing substrate was then added and the reactions were analyzed for formation of splicing intermediates and products as described above. Strains were chosen to represent factors present on different snRNP components or that act at different steps in the assembly and function of the spliceosome. Extracts were made from *spp2-1*, *prp3-1*, *prp6-1*, *prp8-1*, *prp11-1*, *prp16*, *prp18*, *prp22-1*, *prp24-1* and $U4^{ts}$ strains. Efficient spliceosome assembly requires the function of the *PRP3* (J. Anthony, E. M. Weidenhammer and J. L. Woolford Jr, in preparation), *PRP6* (54), *PRP8* (55), *PRP11* (56–58) and *PRP24* (26) gene products. Mutations in or deletions of any of these genes result in accumulation of pre-mRNA *in vitro*. Spp2p is required after spliceosome assembly for the first

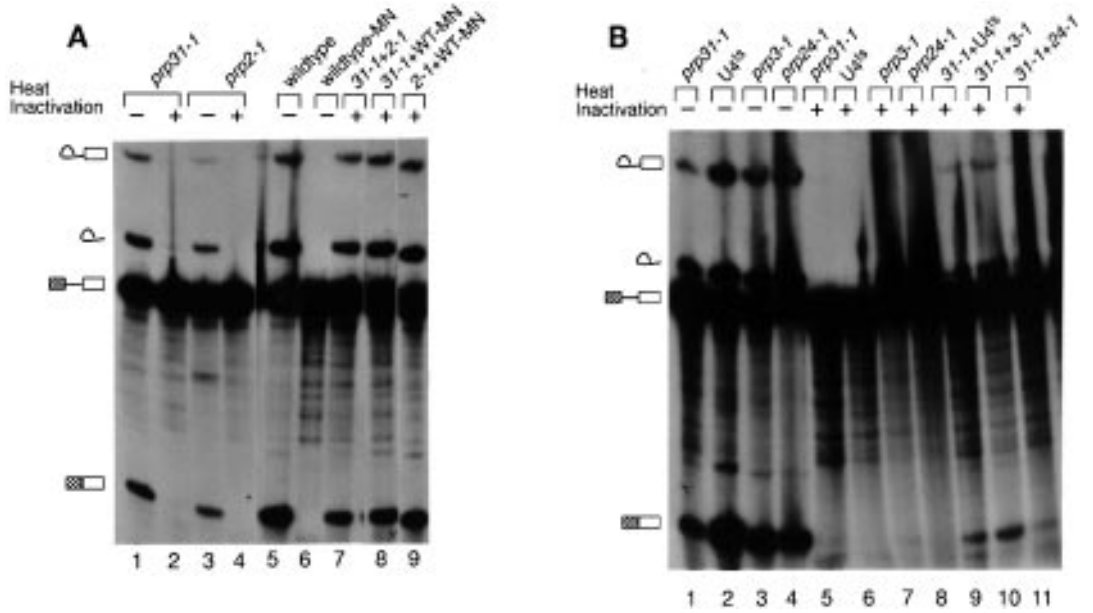

Figure 4. *In vitro* complementation of the temperature sensitivity of *prp31-1*. (**A**) Extracts derived from *prp31-1* (JWY2857) or *prp2-1* (JWY657) strains were maintained on ice (lanes 1 and 3 respectively) or incubated at 37°C for 30 min (lanes 2 and 4). Extracts from wild-type strain BJ2158 were incubated with (lane 6) or without (lane 5) micrococcal nuclease at 30°C for 10 min. Radiolabeled actin pre-mRNA was then added and splicing was allowed to proceed. For complementation assays (lanes 7–9) equal volumes of micrococcal nuclease-treated or heat-inactivated extracts were mixed prior to substrate addition; assays were then carried out as above. Reactions were subjected to denaturing gel electrophoresis to separate splicing intermediates and products. , pre-mRNA; , lariat intron–3′ exon intermediate; , lariat intron product; , spliced mRNA. (**B**) Extracts derived from *prp31-1*, *prp3-1* (JWY630), *prp24-1* (JWY806) or *snr14* ($U4^{ts}$, JWY2419) strains were maintained on ice (lanes 1–4) or heat inactivated (lanes 5–8) prior to addition of radiolabeled splicing substrate. Pairwise complementation assays are demonstrated in lanes 9–11.

cleavage reaction (32), while Prp16p, Prp18p and Prp22p are required at later stages of splicing (59–61). The strain bearing the $U4^{ts}$ mutant allele was identified in the same screen that identified the *prp31-1* strain (35). Integration and mapping analyses indicated that the temperature sensitivity of the strain was due to a mutation in the *SNR14* gene, which encodes U4 snRNA (J. Roy and J. L. Woolford Jr, unpublished observations). Each of these heat-inactivated extracts was able to complement the *in vitro* splicing defect of a heat-inactivated *prp31* extract. Examples of this complementation are shown in Figure 4B. Lanes 1–4 show the *in vitro* splicing activity of extracts derived from a *prp31-1*, a *prp3-1*, a *prp24-1* and a $U4^{ts}$ strain; in lanes 5–8 the extracts were heat-inactivated prior to substrate addition. Equal volumes of the heat-inactivated extracts were combined, splicing substrate was added and the reaction mixes were analyzed for formation of intermediates and products. Lanes 9–11 demonstrate that all combinations of heat-inactivated extracts were capable of complementing the *prp31-1* defect. Similar results were obtained with extracts derived from each of the other mutant strains.

To further examine potential interactions between the *PRP31* gene and other *PRP* genes high copy suppression analysis was utilized. Suppression of a mutant phenotype by overexpression of an unlinked gene is indicative of potential interactions or functional relationships between two gene products (for a review see 62). Such interactions have been defined between *PRP3* and *PRP4* (63), *PRP2* and *SPP2* (63,32) and *PRP21* and *PRP9* (57). The *PRP31* gene was cloned into the 2 μ high copy plasmid vector pRS426. This construct was introduced into *prp 2-prp6*, *prp8*, *prp9*, *prp11*, *prp16*, *prp17-prp27*, *prp32* and *prp34* strains and growth of the transformants was monitored at 23, 30, 32, 35 and 37°C. In all cases increased dosage of the *PRP31* gene had no effect on the temperature sensitivity of these strains (data not shown). In addition, the *PRP18* and *PRP28* genes were introduced into a *prp31-1* strain on a high copy plasmid vector. Again, no suppression of the temperature-sensitive defect was observed.

Synthetic lethality is another indication of interactions between gene products involved in a common cellular pathway (for a review see 62). Combinations of mutant alleles of several genes encoding splicing factors exhibit synthetic lethality, including: *prp3-1* with *prp4-1* (65); *prp24-1* with *prp28-1* (27); various combinations of *prp5*, *prp9*, *prp11* and *prp21* mutant alleles with each other and with mutant U2 snRNA (57,58); *mud1* and *mud2* with mutant U1 snRNA (33); mutant alleles of the *SLU* genes with mutant U5 snRNA and with alleles of the *PRP16* and *PRP18* genes (34). The *prp31-1* allele was tested for synthetic lethality with *spp2-1*, *prp3-1*, *prp4-1*, *prp8-1*, *prp18*, $U4^{ts}$ and *prp24* mutant alleles. In all cases double mutant spore clones were obtained, indicating that the *prp31-1* mutation does not exacerbate the mutant phenotypes of the other genes tested.

Although these analyses with Prp31p have not yet revealed associations of the protein with other splicing factors, such interactions cannot be ruled out based on the negative results described here. High copy suppression is likely to occur in an allele-specific manner. Furthermore, only those mutant proteins that result in reduced levels of activity may be suppressible. Mutant factors that completely obstruct progression of the splicing pathway are not likely to be suppressible by overexpression of interacting factors. Similarly, synthetic lethality and *in vitro* non-complementation of mutant alleles of genes required for splicing is likely to be observed under conditions in which the mutations affect a domain of the components required for interaction. More open ended genetic selections for suppressors

of *prp31* alleles or screens for mutations synthetically lethal with *prp31* may yield information about factors with which Prp31p is associated. Isolation of alleles of the *PRP31* gene bearing mutations in different regions of the sequence may yield substrates that are differentially affected in protein–protein and/or protein–RNA interactions and so would be useful for further defining the requirement for this protein in splicing.

Experiments to determine the potential snRNP association of Prp31p, as well as to define the function of this product in splicing, are in progress.

ACKNOWLEDGEMENTS

We are grateful to Dr Craig Peebles and members of our laboratory for critical comments on this manuscript. This work was supported by Public Health Service grant GM-38782 from the National Institutes of Health to J.L.W. Computer analyses accessed through the Pittsburgh Supercomputing Center were funded by grant DMB 890100P.

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