

A method for rapid mapping of mutations by plasmid rescue strategy in *Saccharomyces cerevisiae*

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MS received 30 December 1995

Abstract. The products of *PRP17* and *PRP18* genes are required for the second step of pre-mRNA splicing reactions in *Saccharomyces cerevisiae*. Temperature-sensitive mutants at either of these loci accumulate products of the first splicing reaction at nonpermissive temperature. To characterize functional regions in these proteins the mutations in three temperature-sensitive alleles of *PRP17* and two temperature-sensitive alleles of *PRP18* were mapped by the plasmid rescue strategy. One of the procedures adopted in the past is plasmid rescue of the mutant allele followed by sequencing of the entire gene. In this work we describe an adaptation of the above procedure that allows, first, rapid mapping of chromosomal segments bearing the mutations, followed by sequence characterization of the minimal segment. The strategy adopted was to integrate a wild-type copy of the gene at the homologous mutant chromosomal locus, followed by recovery of the chromosomal fragments from these integrants as plasmids in *E. coli*. The recovered plasmids were screened by a complementation assay for those that contained in them the chromosomal mutation. The mutations in all the three alleles of *PRP17* map to a small region in the N-terminal half of the protein, whereas the temperature-sensitive mutations in the two alleles of *PRP18* map to different regions of the *PRP18* protein. The recovered mutant plasmids from all five alleles at the two loci were sequenced and the nucleotide changes were found to result in missense mutations in each case. Our strategy is therefore a rapid method to map chromosomal mutations and is of general use in structure–function analysis of cloned genes.

Keywords. *PRP17*; *PRP18*; conditional mutations; plasmid rescue; yeast.

1. Introduction

Mutationally induced phenotypic alteration is a powerful way to isolate and define new genes and study their properties. This is specially useful in model systems for dissection or delineation of biological pathways. While reverse-genetic approaches are often used to study the effect of *in vitro*-generated mutations in cloned genes, mutations generated by classical methods of mutagenesis (chemical, insertional or radiation mutagenesis) remains the method of choice in model systems. The principal advantage is that no prior knowledge about the genes or their products is required, and the only parameter to be decided upon before generating the mutants is the phenotypic alteration that is to be screened for. Once a mutant phenotype is obtained the facile methods now available for cloning wild-type genes based on complementation of the mutant phenotype allow structural analysis of the cloned genes and their predicted products. In many cases understanding structure–function

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relations of products of cloned genes is aided by the presence of conserved motifs that help to arrive at a deduced biochemical function for the protein. In other cases, where the gene product in question is unique, chromosomal mutations that result in altered functions can be of use in deducing the functional region/regions of the protein. In the yeast *Saccharomyces cerevisiae* the latter approach is particularly amenable to analysis. Insertion of the cloned gene into the mutant chromosome by homologous recombination followed by recovery of the chromosomal mutation on a plasmid has been a method exploited to study *in vivo*-generated mutations. We have extended the use of this strategy to map temperature-sensitive (ts) mutations in two yeast genes, *PRP17* and *PRP18*. The two genes chosen are required for the second step of pre-mRNA splicing reactions in *Saccharomyces cerevisiae* (Vijayraghavan and Abelson 1989; Vijayraghavan *et al.* 1989). The ts mutations result in accumulation of products of the first splicing reaction, exon 1 and lariat intermediate, at nonpermissive temperature (Vijayraghavan and Abelson 1990). While both these genes are required at the same reaction step in pre-mRNA splicing, they encode unique genes with no homology to each other. The predicted Prp17p has conserved repeat units found among the members of a group of proteins that are similar to the G-beta subunit of the signal transducing protein bovine transducin (Company and Abelson, unpublished observation; Seshadri *et al.* 1996). *PRP18* on the other hand codes for a unique protein with no conserved structural motifs (Horowitz and Abelson 1993). We have modified the plasmid rescue strategy (Roeder and Fink 1980; Winston *et al.* 1983) to rapidly define regions of these two proteins that may be essential for function in pre-mRNA splicing by first mapping and then determining the nature of the mutation in each of the ts alleles at the two loci.

2. Materials and methods

2.1 Strains and growth media

E. coli DH5 α cells were used for cloning and plasmid amplifications. *E. coli* transformations were done by electroporation (Dower *et al.* 1988) using a BioRad electroporator. Yeast strains were maintained on standard media as described by Sherman *et al.* (1986). The ts strains were maintained at 23°C, and the nonpermissive temperature used was 37°C. The *prp17* and *prp18* ts alleles described here are listed in table 1. To generate haploid *prp18 leu⁻* strains, ts mutants were crossed to wild-type strains *SEY6210* or *SEY6211*, and the resulting diploids allowed to sporulate. Random spores from these diploids were screened for temperature sensitivity (*prp18*) and prototrophy for leucine (*leu⁻*).

2.2 Yeast transformations

Yeast transformations were done by lithium acetate procedure (Ito *et al.* 1983) with the minor modification of growing ts strains at 23°C and using 37°C for heat-shock treatment. The transformations were done with linearized plasmids so as to direct homologous integration to the respective chromosomal loci. Transformants were selected on appropriate drop-out media at 23°C (Sherman *et al.* 1986). For

Table 1. List of strains.

Strain	Genotype	Source/Reference
<i>E. coli</i> DH5 α	<i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1 supE44 Δlac419</i> (ϕ 80 <i>lacZ</i> Δ M15)	Hanahan 1983
<i>S. cerevisiae</i>		
<i>prp17-1</i>	<i>MATα lys2-801 ade2-101 ura3-52 his3Δ200</i>	Vijayraghavan <i>et al.</i> 1989
<i>prp17-2</i>	<i>MATα lys2-801 ade2-101 ura3-52 his3Δ200 tyr1</i>	Vijayraghavan <i>et al.</i> 1989
<i>prp17-3</i>	<i>MATα lys2-801 ade2-101 ura3-52 his3Δ200 trp1Δ63 leu2,3-112</i>	Frank <i>et al.</i> 1992
<i>prp18-2</i>	<i>MATα lys2-801 ade2-101 ura3-52 his3Δ200 tyr1 leu2,3-112</i>	Vijayraghavan <i>et al.</i> 1989
<i>prp18-3</i>	<i>MATα lys2-801 ade2-101 ura3-52 his3Δ200 leu2,3-112</i>	Vijayraghavan <i>et al.</i> 1989
SEY6210	<i>MATα lys2-801 ura3-52 his3Δ200 trp1Δ901 leu2,3-112</i>	S. Emr
SEY6211	<i>MATα ade2-101 ura3-52 his3Δ200 trp1Δ901 leu2,3-112</i>	S. Emr

complementation analysis the colony-purified transformants were checked for ability to grow at nonpermissive temperature, i.e. 37°C.

2.3 Deletion clones and sequencing

Standard methods were used for DNA restriction and ligation (Sambrook *et al.* 1989). The noncomplementing rescued plasmids from the *ts prp18-2* (p18-2BglIII) and *prp18-3* (p18-3XhoI) mutants were used to generate deletion clones. Plasmids p18-2BglIII and p18-3XhoI were digested with *EcoRV* and *XhoI* (see figure 5 for the relative positions of these sites), filled by Klenow enzyme, and self-ligated. The ligation mix was used to transform *E. coli* and the plasmids with the region between *EcoRV* and *XhoI* sites deleted were recovered. These plasmids (del-p18-2BglIII and del-p18-3XhoI) containing the *prp18* gene with the chromosomal mutation were used in sequencing reactions with the Sequenase kit (USB) and α^{35} S-dATP from BRIT (Bombay) following the manufacturer's protocol.

The oligonucleotides used for sequencing *prp17* alleles were:

A1, 5' TTCTCCAGATGCCATGGGTTTAGT 3'
 Sn1, 5' GCAGTGGTTCCTTCTGGA 3'
 Sb1, 5' AAGTCAGAACTAAAAAGAAGAAGACGG 3'

3. Results

3.1 Recovery of chromosomal *ts prp17* alleles as plasmids

A 3.3-kb *BamHI-SalI* genomic fragment of wild-type *PRP17* was cloned into the yeast integrating vector pRS303 which has the yeast *HIS3* gene as selectable marker (Sikorski and Hieter 1989). The p17RS303 clone was linearized at the unique *BstEII* site in the *PRP17* DNA. Transformations of *prp17-1*, *prp17-2* and *prp17-3* strains were done with 10 μ g of linear plasmid DNA. Homologous integration at the *prp17* locus should create a gene duplication with one wild-type and one mutant copy separated by plasmid sequence (figure 1). Such homologous integration of the plasmid-borne *PRP17* into the *prp17-1*, *prp17-2* and *prp17-3* loci was confirmed

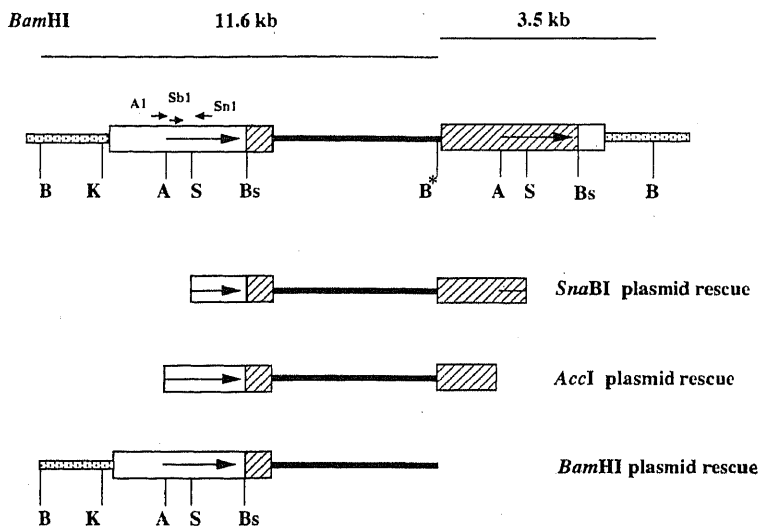


Figure 1. Map of the *prp17* genomic locus after integration of plasmid-borne wild-type *PRP17*. The chromosomal *prp17* is shown as an open box, the plasmid-borne wild-type copy as hatched box, and plasmid pRS303 sequence as a thick solid line. The expected *Bam*HI fragments that will be produced from integrants with a single copy of plasmid are indicated above the map as 11.6-kb and 3.5-kb fragments. Maps of the expected fragments produced on digestion of genomic DNA from such integrants with *Sna*BI, *Acc*I and *Bam*HI are given below. These fragments upon recircularization give the rescued plasmids that include different extents of the mutant *prp17* allele. Restriction enzymes are: B, *Bam*HI; A, *Acc*I; S, *Sna*BI; Bs, *Bsr*EII; K, *Kpn*I; B*, *Bam*HI site in the polylinker of plasmid pRS303.

by Southern analysis. Genomic DNA from the transformants, prepared by the method of Poliana and Adam (1991), was used for Southern analysis. Southern blots of genomic DNA from single-copy integrants at the *prp17* locus, when digested with *Bam*HI and probed with a fragment of the *PRP17* gene, should give a 11.6-kb and a 3.5-kb fragment (as depicted in figure 1). On the other hand an integrant with multiple copies of the plasmid at the *prp17* locus will yield an additional hybridizing fragment of 7.8 kb that corresponds to the unit plasmid length of p17RS303. Both single-copy and multicopy integrants were obtained after integrative transformation of all three *prp17* strains (figure 2: single-copy integrants are in lane 2 for *prp17-1*, lane 4 for *prp17-2* and lane 5 for *prp17-3*; multicopy integrants are lane 1 for *prp17-1* and lane 3 for *prp17-2*). The integrants with a single copy of *PRP17* at each of the three *prp17* loci were chosen for further analysis. Genomic DNA from each of these integrants was digested with *Bam*HI, *Acc*I or *Sna*BI for release of linear fragments that can be recovered as plasmids in *E. coli* (figure 1 schematically shows the predicted restriction fragments from the *prp17* loci of these integrants). In all the digests, the linear fragment produced will contain plasmid sequence and the complete gene for Prp17p but with segments of the gene derived from the genomic mutant *prp17* locus. These linear fragments were self-ligated at low DNA concentrations (2 µg/ml), and the resulting plasmids were recovered in *E. coli* after electroporation. If the recovered gene contains a segment of the

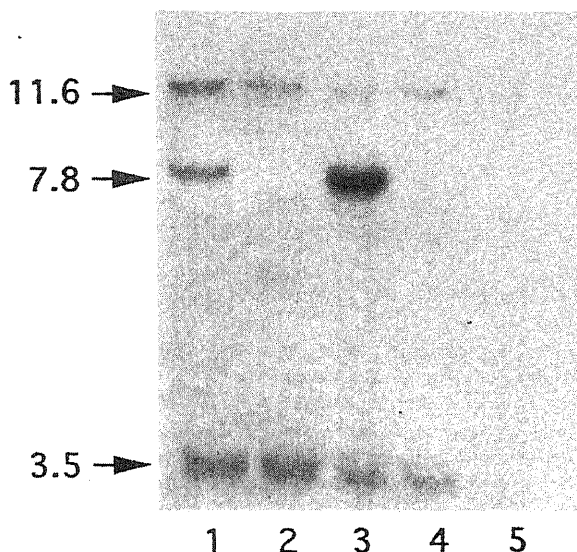


Figure 2. Southern blot for identification of integrants with a single copy of *PRP17* at the mutant chromosomal locus. 2 µg of genomic DNA from several integrants was digested with *Bam*HI, electrophoresed, Southern blotted, and probed with a 2.0-kb fragment of *PRP17*. Lanes 1 and 2 contain digests of genomic DNA from two different integrants at *prp17-1*; lane 1 identifies a multicopy integrant whereas lane 2 identifies a single-copy integrant. Lanes 3 and 4 contain digests of genomic DNA from a multicopy and a single-copy integrant respectively at *prp17-2*. Lane 5 contains a digest that identifies a single-copy integrant at *prp17-3*.

genomic *prp17* locus with the *ts* mutation then the plasmid will not complement the same mutation. On the other hand if in the recovered plasmid the fragment from the genomic locus contains wild-type sequence then it should be capable of complementing the *ts* phenotype. The *Bam*HI, *Acc*I and *Sna*BI rescued plasmids from *prp17-1*, *prp17-2* and *prp17-3* were used to transform the *ts prp17* strains, and complementation of temperature sensitivity was analysed. All the plasmids recovered from the *Sna*BI digests were capable of complementing the *ts* phenotype as seen by growth at 37°C of the transformed strains (figure 3, column S). On the other hand the plasmids recovered from *Acc*I and *Bam*HI digests were unable to complement the *ts* phenotype (figure 3, columns A and B at 37°C). These results indicate that the *ts* mutations in *prp17-1*, *prp17-2* and *prp17-3* were recovered in *Bam*HI and *Acc*I rescued plasmids, while wild-type sequence was recovered in the *Sna*BI rescued plasmids. Therefore our analysis mapped the *ts* mutations to the 370-bp region between the *Acc*I and *Sna*BI sites of the *prp17* loci. This region corresponds to a domain in the N-terminal half of the predicted Prp17 protein. The *Acc*I rescued plasmids from the three *prp17* strains were sequenced in this region to determine the nature of the mutations. The oligonucleotides A1, S_n1 and S_b1 were used as primers for sequencing (positions of the oligonucleotides indicated in figure 1). The mutation in *prp17-1* is a G-to-C transversion (figure 4a) resulting in a change of Gly (127) to Ala; in *prp17-2* an A-to-G transition (figure 4b) results

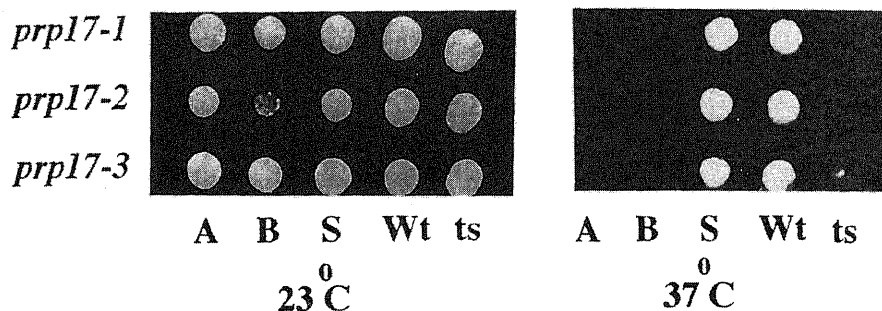


Figure 3. Complementation profiles of *prp17* alleles obtained after transformation of ts strains with the various plasmid-rescued clones. The left panel shows growth at 23°C and the right panel shows growth at 37°C. Column A, growth of transformants containing the *AccI* rescued plasmids recovered from the *prp17-1*, *prp17-2* and *prp17-3* strains; column B, growth of transformants containing the *Bam*HI rescued plasmids; column S, growth of transformants containing the *Sna*BI rescued plasmids recovered from the three strains; column Wt, growth of transformants with wild-type *PRP17*; column ts, temperature sensitivity of the parent *prp17* mutations.

in a change of Arg (58) to Gly; and in *prp17-3* a C-to-T change (figure 4c) results in a change of Ser (54) to Leu.

3.2 Mapping of *ts* mutations in *prp18* alleles

We followed the plasmid rescue strategy described in the previous section to map the mutations in the temperature-sensitive *prp18* alleles also. The 2.5-kb *Bam*HI-*Sal*II fragment containing the wild-type *PRP18* gene was subcloned into the yeast integrating vector pRS305 which has the yeast *LEU2* gene as selectable marker (Sikorski and Hieter 1989). *prp18-2 leu2,3-112* and *prp18-3 leu2,3-112* haploid strains were constructed as described in Materials and methods. The p18RS305 clone was linearized at a unique *Hind*III site in the *PRP18* genomic sequence, and 10 µg of linearized plasmid was used for integrative transformation of the two *ts prp18 leu*⁻ strains. Integration at the *prp18* locus will result in gene duplication with one mutant and one wild-type copy separated by plasmid sequence (figure 5). Integrants with a single copy of the wild-type *PRP18* were identified by Southern analysis. Genomic DNA from several integrants was double-digested with *Nru*I and *Sal*II, Southern blotted, and probed with a 700-bp fragment of *PRP18*. Integrants with a single copy of the plasmid give a 2.5-kb and a 17-kb hybridizing fragment as expected (figure 6, lane 1 and lane 2 for single-copy integrant at *prp18-2* and *prp18-3* respectively). Integrants with multiple copies of the plasmid at either of the *prp18* loci give an additional hybridizing fragment of 8 kb (figure 6, lanes 3 and 4 show multicopy integrants for *prp18-2* and *prp18-3*). Genomic DNA from single-copy integrants was taken for recovery of the chromosomal mutations as plasmids. Genomic DNA from these single-copy integrants was digested with *Bgl*III or *Xho*I (figure 5 shows the predicted restriction fragments that should be generated). The linear fragments produced were self-ligated at low DNA concentrations (2 µg/ml) and the resulting plasmids recovered in *E. coli*. The recovered plasmids were

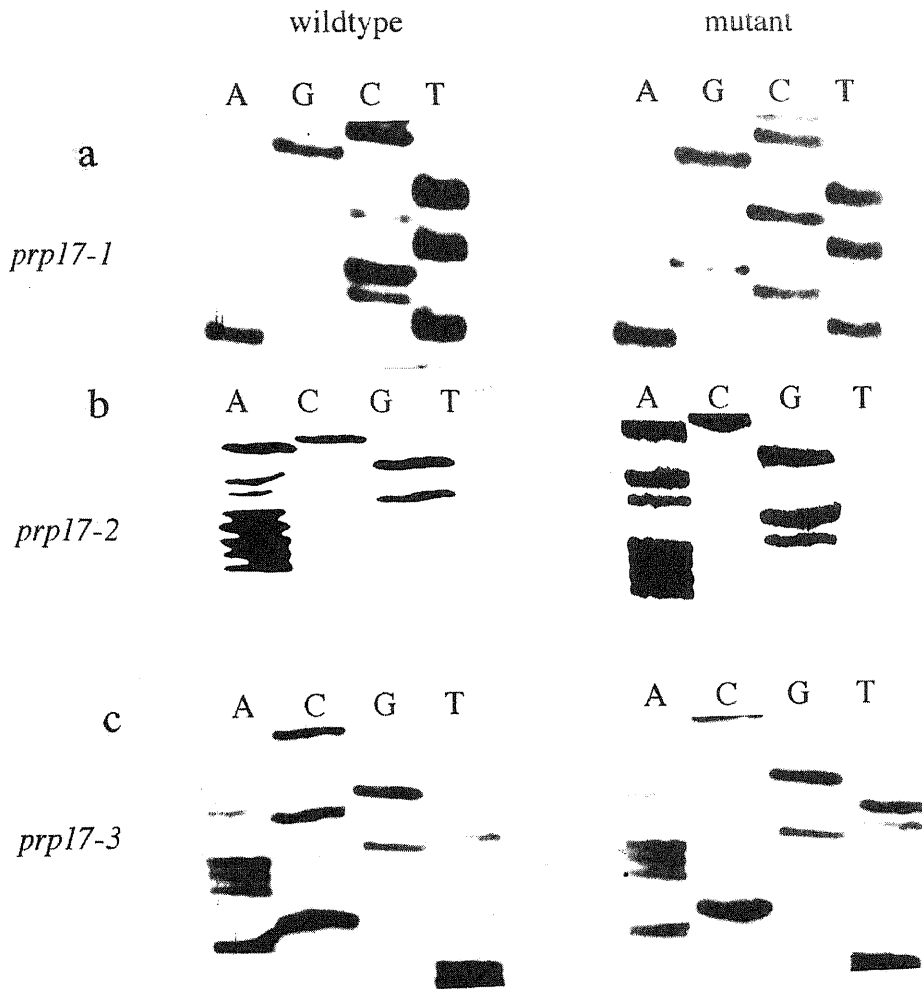


Figure 4. Sequence analysis of the mutations in the three *prp17* alleles. (a) A sequencing reaction done with primer Sb1 that reads the noncoding strand; the mutation is a C-to-G change which corresponds to a G-to-C transversion in the sense strand of the *prp17-1* allele. (b) A region in *prp17-2* sequenced with primer A1 which reads the sense strand; the mutational change here is an A-to-G transformation. (c) The mutant region in *prp17-3* allele sequenced with primer A1; the mutation in this case is a C-to-T conversion.

analysed for complementation of the ts *prp18* mutations. In the case of *prp18-2* the plasmid recovered from the *Bgl*III digest was unable to complement the ts phenotype (figure 7, sector Bg1 at 23°C versus 37°C) whereas the plasmid recovered from the *Xho*I digest could rescue the ts phenotype of *prp18-2* (figure 7, sector X1 at 23°C and 37°C), indicating that the ts mutation lies between the *Bgl*III and the *Hind*III sites of the *prp18-2* locus. In the case of *prp18-3* the plasmid recovered from the *Bgl*III digest complemented the ts phenotype of *prp18-3* (figure 7, sector Bg2 at 23°C and 37°C) whereas the plasmid recovered from the *Xho*I digest was unable to complement the ts mutation (figure 7, sector X2 at 23°C versus 37°C), indicating that the ts mutation in *prp18-3* lies between the *Hind*III and the *Xho*I

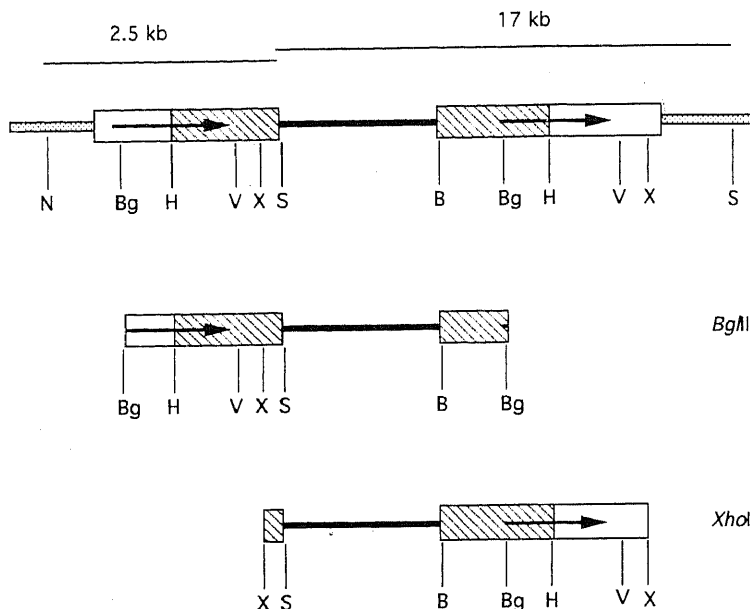


Figure 5. Map of the *prp18* genomic locus after integration of plasmid-borne *PRP18*. The chromosomal *prp18* locus is shown as an open box, the plasmid-borne wild-type copy as hatched box, and plasmid pRS305 sequence as a thick solid line. The expected *NruI* and *SalI* fragments that will be produced from integrants that have a single copy of the plasmid are indicated above the map as 17-kb and 2.5-kb fragments. The maps of the expected fragments produced upon digestion of genomic DNA from such integrants with *BglII* and *XhoI* are given below. These fragments upon recircularization give the rescued plasmids. Restriction enzymes are: B, *BamHI*; Bg, *BglII*; V, *EcoRV*; N, *NruI*; S, *SalI*; X, *XhoI*; H, *HindIII*.

sites of the *prp18-3* locus. Therefore the mutations in *prp18-2* and *prp18-3* map to different regions of Prp18p. The DNA segment between *BglII* and *HindIII* sites in the plasmid p18-2*BglII* and the DNA segment between *HindIII* and *EcoRV* sites in p18-3*XhoI* were sequenced. To facilitate sequencing of these segments containing the mutations the segment between *EcoRV* and *XhoI* (see figure 5 for the relative positions of the sites in *PRP18* gene), which contains the 3' untranslated region, was deleted from p18-2*BglII* and p18-3*XhoI*, as described in Materials and methods. These deletion derivatives containing the chromosomal mutation were sequenced to determine the nature of the mutation. In *prp18-2* the mutational alteration was a C-to-G transversion resulting in a change of Ser(39) to Cys, and in *prp18-3* a T-to-A transversion results in a change of Val(180) to Asp (table 2).

4. Discussion

In *Saccharomyces cerevisiae* many genes involved in various biochemical and cellular pathways have been identified through genetic screens of temperature-sensitive mutants for defects in a particular function. The advantage of using such a classical

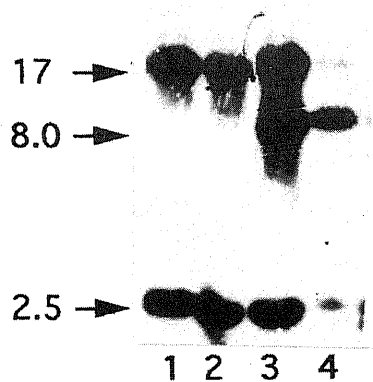


Figure 6. Southern blot for identification of integrants with a single copy of *PRP18* after homologous recombination. 2 μ g of genomic DNA from several integrants was double-digested with *Nru*I and *Sal*I, electrophoresed, Southern blotted, and probed with a 700-bp fragment of *PRP18*. Lanes 1 and 3 contain digests of genomic DNA from two different integrants at *prp18-2*; lane 3 identifies a multicopy integrant whereas lane 1 identifies a single-copy integrant. Lanes 2 and 4 contain digests of genomic DNA from a single-copy and a multicopy integrant respectively at the *prp18-3* locus.

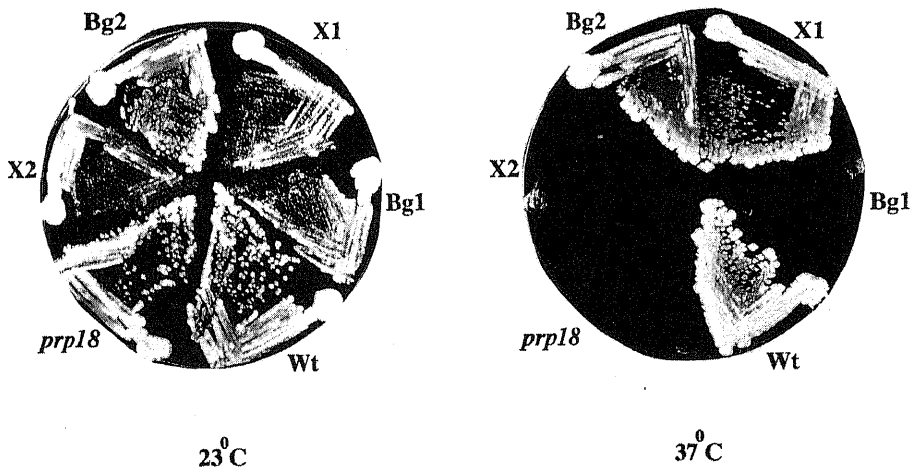


Figure 7. Growth of transformants obtained after introduction of the plasmids recovered from the *prp18* strains. The plate on the left shows growth at 23°C and the one on the right growth at 37°C. Sectors Bg1 and X1 are *prp18-2* transformants with *Bgl*III and *Xho*I rescued plasmid DNA respectively; these plasmids were recovered from *prp18-2*. *Bgl*III rescued plasmid from *prp18-2* does not complement *prp18-2* ts mutation (sector Bg1 at 37°C) while the *Xho*I rescued plasmid complements the ts phenotype (sector X1). Sectors Bg2 and X2 are *prp18-3* transformants with *Bgl*III and *Xho*I rescued plasmids respectively that were recovered from *prp18-3*. The *Bgl*III rescued plasmid complements the ts phenotype of *prp18-3* (sector Bg2 at 37°C) but the *Xho*I rescued plasmid does not complement this phenotype (sector X2).

Table 2. Complementation profile of *prp18* alleles by the plasmid-rescued clones.

<i>prp18</i> allele	Rescued plasmid*	Complementation of <i>prp18</i> at 37°C†	ts mutation‡
<i>prp18-2</i>	<i>Bgl</i> III	-	S 39 C
	<i>Xho</i> I	+	
<i>prp18-3</i>	<i>Bgl</i> III	+	V 180 D
	<i>Xho</i> I	-	

*The rescued plasmids from the single-copy integrants at *prp18-2* and *prp18-3* loci are listed here; the nomenclature of the plasmids reflects the enzyme digests done for recovery of the plasmids.

†The plasmid DNA indicated in column 2 was used to transform *prp18-2* or *prp18-3* mutants and growth of the transformants at 37°C is indicated.

‡Amino-acid changes in the *prp18* alleles determined by sequence analysis. The amino acids are given in single-letter code, the wild-type amino acid first, followed by the position of the amino acid in Prp18p, and then the mutant amino acid.

screen for delineating cellular pathways is that it can be performed without any knowledge about the molecular nature of the genes involved. Isolation of conditional mutations in genes of interest has also been of use in elucidating functional domains in proteins. The facile molecular-genetic techniques developed in *Saccharomyces cerevisiae* allow cloning of wild-type and mutant genes for studying structure-function relations. For analysis of mutant gene products it is necessary to recover the mutant chromosomal alleles. Classically the mutant alleles have been recovered and mapped in yeast by the gap repair strategy. Briefly, in this method a cloned, plasmid-borne copy of the wild-type gene is used to recover chromosomal mutations. Segments of the wild-type gene are deleted in the plasmid-borne copy by the use of restriction enzymes. Several deletions are generated that span the entire cloned locus in an overlapping fashion. These plasmids bearing deletions of the wild-type gene are used to transform yeast strains with mutations in the corresponding chromosomal locus. Through *in vivo* homologous pairing and repair mechanisms the gaps/deletions in the plasmid-borne gene are repaired with sequence information from the chromosomal allele. If the gapped region in the plasmid corresponds to a region of the chromosomal gene that contains the mutation then the mutation will be recovered on the plasmid (Weaver *et al.* 1983). One of the several genes for which this strategy was adopted is *RNA12*, a gene required for pre-rRNA maturation (Liang *et al.* 1992). Here a dominant ts mutation was recovered in a plasmid and then identified by sequence analysis. This strategy requires considerable *in vitro* manipulation to generate gaps in the plasmid-borne wild-type allele that span the whole gene, and therefore has limitations. In the plasmid rescue method described here for mapping chromosomal mutations, the *in vitro* manipulations are fewer. The requirements are a unique restriction site for the homologous integration of the wild-type gene into the mutant locus and presence of convenient restriction sites for the rescue of segments of the chromosomal locus as plasmids. The plasmid rescue strategy has been used before to recover mutant alleles in yeast (Roeder and Fink 1980). We have modified this method of plasmid rescue to rapidly map temperature-sensitive mutations in three *prp17* alleles and two *prp18* alleles. The ts mutations in the *prp17* alleles map to a small 370-bp region that corresponds

to the N-terminal half of the predicted Prp17p. Thus the three temperature-sensitive *prp17* alleles define an N-terminal domain of the protein that may be necessary for function. Two of the mutations map very close to each other, only four amino acids apart, possibly indicating the functional significance of this region of the protein. In ongoing studies on structure-function relations in Prp17p we have further characterized this domain in the N-terminal region of Prp17p and find that it interacts with other factors required for the second step of splicing (Seshadri *et al.* 1996). In the case of *prp18* the two temperature-sensitive mutations map to different regions of Prp18p, suggesting one of two possibilities. One is that the number of alleles available is insufficient to define functional domains, and the alternative possibility is that the conditional mutations do not delineate functional domains in Prp18p, possibly because it acts as a single-domain protein. Characterization of new *prp18* alleles will shed light on domain architecture of Prp18p. Finally the method described here, which recovers chromosomal mutations as plasmids, could be of wider application in any system/organism where integration of a cloned gene to the homologous chromosomal locus is possible.

Acknowledgements

We thank Imran Siddiqi and K. VijayRaghavan for their useful suggestions, and Christine Guthrie for providing the temperature-sensitive *prp17-3 (slu4-1)* strain. We acknowledge the DBT-supported facility at Centre for Genetic Engineering, Indian Institute of Science, for the synthesis of the oligonucleotides used in this study. This work was supported by a grant from Department of Biotechnology, Government of India, to Usha Vijayraghavan.

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