A mutational analysis of the yeast protein splicing factor, Prp2.

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Declaration

I hereby declare that I alone have composed this thesis and that, except where stated, the work presented herein is my own.

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Abstract

The Prp2 protein of *Saccharomyces cerevisiae* is a 100kDa non-snRNP splicing factor with amino acids common to the DEAD/H-box family of ATP-dependent RNA helicases. Prp2, Prp16 and Prp22 proteins have extensive sequence similarity, and function at sequential steps in the splicing pathway. Prp2 and Prp16 form transient reactions with the spliceosome, Prp2 at step 1 and Prp16 at step 2.

In addition to the conserved motifs of the DEAD/H-box family, Prp2 also contains a sequence resembling a zinc finger motif. This sequence has been extensively mutagenised, and two mutations were found by *in vivo* complementation assays to affect the function of Prp2.

In order to study the molecular interactions of Prp2 at step 1, several dominant negative *PRP2* alleles had been generated previously in this laboratory by site-directed mutagenesis. When overexpressed from an inducible promoter, these cause accumulation of pre-mRNA in the presence of wild-type *PRP2*. These dominant negative alleles and wild-type *PRP2* were subjected to deletion analyses. In the case of one dominant negative mutant which has a GKT to AKT change in the ATP-binding motif, some dominant activity was retained despite the removal of 19kDa from the protein.

It has been suggested that the Prp16 protein plays a proofreading role in splicing, as a mutant of *PRP16*, *prp16-1*, suppresses an A-C change of the branch nucleotide. A mutation analogous to the *prp16-1* allele was created in *PRP2* to determine if Prp2 plays a role in influencing the fidelity of splicing. Although no effect was found for this and other mutants of *PRP2*, it was discovered that overexpression of wild-type *PRP16* had an effect similar to that of the *prp16-1* mutant on the fidelity of splicing.

A new hypothesis is proposed for the mechanism of suppression of branchpoint mutants by Prp16 whereby the Prp16 protein might mediate the binding of an unknown proofreading factor to the branchpoint.

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Abbreviations.

amp	ampicillin
ADP	adenosine 5' diphosphate
APS	ammonium persulphate
ATP	adenosine 5' triphosphate
bp	base pair
BSA	bovine serum albumin
°C	degrees Celcius
Ci	Curie
D	Dalton
dATP	2'-deoxyadenosine 5' triphosphate
dCTP	2'-deoxycytosine 5' triphosphate
dGTP	2'-deoxyguanosine 5' triphosphate
dTTP	2' deoxythymidine 5' triphosphate
dH_2O	distilled water
ddH_2O	double-distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E1, E2	exon 1, exon 2
EDTA	ehylenediamine tetracetic acid
5-FOA	5-fluoroorotic acid
g	gram
GDP	guanosine 5' diphosphate
GTP	guanosine 5' triphosphate
HEPES	4-(2-hydroxylethyl)-1-piperizine
his-tag	poly-histidine tag
IgG	immunoglobulin G
IVS	intervening sequence (intron)
IVS-E2	lariat-intron-exon 2 intermediate
k	kilo (10^6)
l	litre
m	mili (10^{-3})
M	moles per litre
mole	6.02×10^{23} molecules
mRNA	messenger RNA
n	nano (10^{-9})
NTP	nucleotide triphosphate
2'-OH	2' hydroxyl group
3'-OH	3' hydroxyl group
oligo	oligodeoxyribonucleotide
^{32}P	a β-emitting isotope of phosphorous
PAS	protein A sepharose
PEG	polyethylene glycol
pH	negative log of hydrogen ion concentration
pre-mRNA	precursor mRNA
R	purine nucleotide

RNA	ribonucleic acid
Rnase	ribonuclease
Rnasin	ribonuclease inhibitor
RNP	ribonucleoprotein
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
snRNA	small nuclear RNA
snRNP	small nuclear RNP
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
tRNA	transfer RNA
UTP	uridine 5' triphosphate
UV	ultraviolet
V	volt
v/v	volume per unit volume
w/v	weight per unit volume
μ	micro (10^{-6})
X	any amino acid
Y	pyrimidine base

Abbreviations for amino acids

Amino acid	Three-letter abbreviation	One-letter symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Chapter one.

Introduction

1.1 Intervening sequences in eukaryotes.

Many eukaryotic genes are interrupted by intervening sequences or introns. Berget *et al* first proposed in 1977 that introns were spliced out of heterogeneous nuclear RNA (hnRNA) transcripts in the nucleus to produce mature messenger RNA (mRNA). This is the process whereby nuclear pre-mRNA introns are accurately spliced out resulting in mature mRNA and an excised intron with a branched, lariat structure. Conserved sequences in the intron are known to be involved in directing the splicing reaction (Padgett *et al*, 1986) which occurs in a large multicomponent protein/RNA complex known as the spliceosome.

Nuclear pre-mRNA splicing is one of four known mechanisms of RNA splicing and is analogous to the splicing of group II introns. Group II introns are removed via two transesterification reactions which also results in the formation of a branched intron-lariat structure (Cech and Bass, 1986; for recent review, see Michel and Ferat, 1995). Although some introns have been shown to be self-splicing *in vitro* (Peebles *et al*, 1986; Van der Veen *et al*, 1986; Schmelzer *et al*, 1986), most group II introns depend on proteins for *in vivo* activity. As in nuclear pre-mRNA splicing, there are many RNA helices formed and dissociated during the splicing process which are likely candidates for the action of RNA helicase proteins.

The other two classes of intron, transfer RNA (tRNA) introns and group I introns are spliced by different mechanisms resulting in the intron being excised as a linear molecule (Culbertson and Winey, 1989; Cech and Bass, 1986).

In the yeast *Saccharomyces cerevisiae*, very few structural genes are known to have introns, although most ribosomal genes do. All intron-containing structural genes have only one intron, with the exceptions of *MATa1* (Miller, 1984) and *RPL8A* which contain two.

1.2 *Cis*-acting elements.

Comparison of DNA sequences led to the discovery of conserved regions around the 5' cut site and the 3' cut site in mammalian genes. In addition, a run of pyrimidines is found upstream of the 3' conserved region in mammalian introns (Breathnach *et al*, 1978; Mount, 1982). Yeast sequences were shown to agree with the mammalian consensus sequences but with a higher degree of conservation. However, many yeast genes lack the polypyrimidine tract downstream of the branch nucleotide present in higher eukaryotic introns. In addition, yeast introns also had a highly conserved sequence around the branchpoint nucleotide (Leer *et al*, 1984; Teem *et al*, 1984). (Table 1.1)

Table 1.1 Conserved sequences in mammalian and yeast introns.

	5' splice site	branchpoint	3' splice site
Mammalian	$^{c}_{a}$ug/GU$^{A}_{G}$AGU	**UNCURAC**	**(Y)nNAG/g**
Yeast	**/GUAUGU**	**UACUAAC**	**YAG**

Table 1.1 Capital letters indiacte intron sequence and lower case indicates coding sequence.

The higher conservation of these sequences in yeast partly explains why yeast transcripts can be spliced in mammalian systems, while mammalian transcripts cannot be spliced in yeast (see section 1.4). The distance between the branchpoint sequence and the 3' splice-site in mammalian introns, usually between 18 and 40 nucleotides, is important in the selection of the branch nucleotide. In yeast introns, the branchpoint is typically between 20 and 40 nucleotides upstream of the 3' splice-site.

1.3 Mechanisms of splicing.

The first report of the presence of branched RNA in the nucleus (Wallace and Edmonds 1983) suggested that mRNA in the cytoplasm was produced as a result of hnRNA being processed in the nucleus. Branched RNA was proposed to be the cause of a block to

reverse transcriptase in *in vitro* splicing experiments (Ruskin *et al*, 1984). The intron was shown to be spliced out in the form of a lariat with a 2'-5' phosphodiester linkage in a way similar to group II introns (Domdey *et al*, 1984). A conserved adenosine nucleotide in the branchpoint sequence is used in the branch formation in yeast. *In vitro* studies of the splicing pathway showed it to be a two-step process in both mammalian and yeast systems (Zeitlin and Estradiatis, 1984; Rodriguez *et al*, 1984). Step 1 involves nucleophilic attack by the 2' hydroxyl group of the branchpoint adenosine on the 5' splice site 3',5' phosphodiester bond, and concomitant formation of a branched, lariat intermediate with the 5' terminal guanosine of the intron covalently associated by means of a 2',5'-phosphodiester bond to the branch adenosine. Step 2 involves attack by the 3'hydroxyl group of exon 1 on the phosphodiester bond at the 3' splice site, and ligation of the two exons (figure 1.1).

1.4 Mammalian and yeast splicing.

Methods for studying splicing *in vitro* were developed for both mammalian and yeast systems (Ruskin *et al*, 1984; Lin, 1985). It was found that Mg^{2+} ions and ATP were required for splicing to occur (Krainer *et al*, 1984; Lin, 1985). The phosphates in the new phosphodiester bonds are provided by the splice site phosphates of the pre-mRNA and not from exogenous ATP (Padgett *et al*, 1984; Kornarska *et al*, 1985; Lin *et al*, 1985). The ATP involved in splicing is likely to be involved in allowing conformational changes within the spliceosome to occur during the splicing process. The yeast *in vitro* system was shown to be accurate by comparing splicing reactions with those *in vivo*.

Figure 1.1. Mechanism of pre-mRNA splicing.

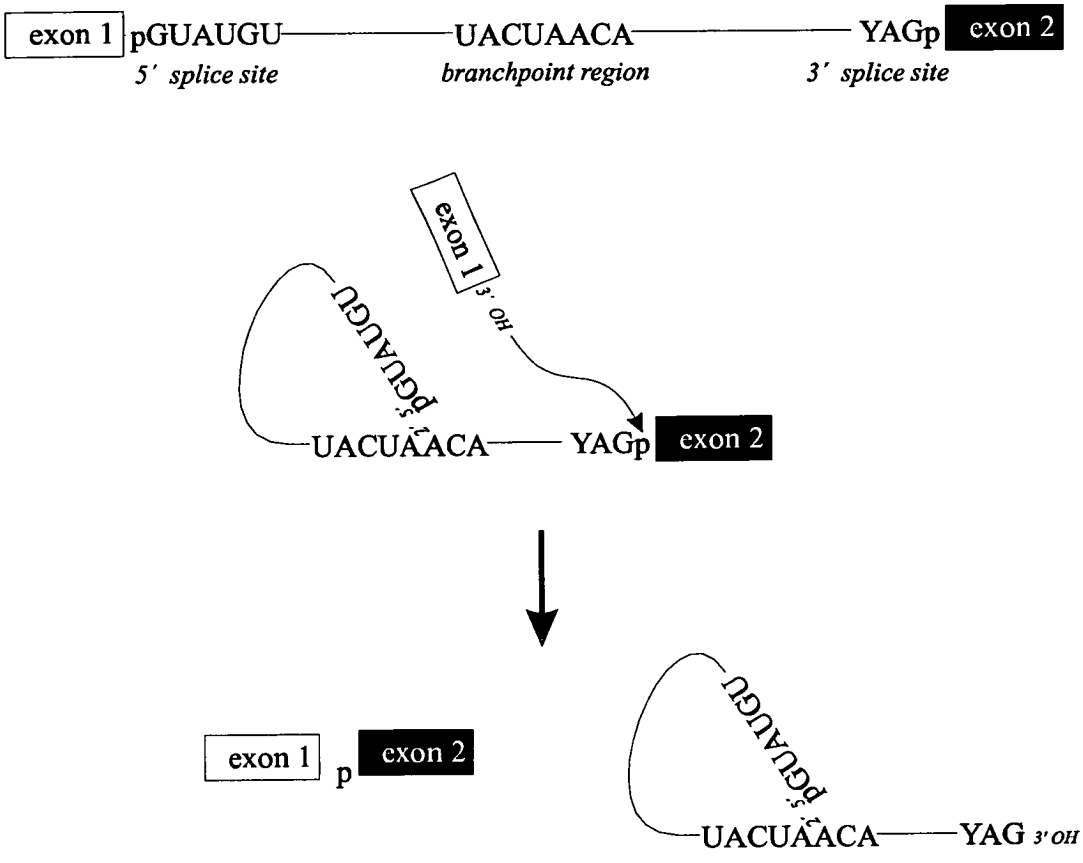


Figure 1.1. Nuclear pre-mRNA splicing consists of two transesterification reactions. The first, step 1, involves cleavage at the 5' splice-site of the intron and formation of the branched lariat-exon 2 intermediate and free exon 1. Step 2, which is ATP-dependent involves cleavage at the 3' splice-site and joining of the two exons to form the mature mRNA. The lariat is subsequently debranched and then degraded.

The development of *in vitro* splicing systems allowed the direct comparison of mammalian and yeast splicing mechanisms. Transcripts which were found to splice normally both *in vitro* and *in vivo* in yeast, were also found to splice in mammalian *in vitro* systems. One difference found was that the branchpoint nucleotide was variable in the HeLa splicing system, both between and within introns, and functionally constrained in yeast. Further to this, when the usual branchpoint sequence was removed from the transcript, splicing still occurred in HeLa extracts using an alternative branchpoint nucleotide which cannot be used by the yeast system (Ruskin *et al*, 1986). Plant introns have also been shown to splice in mammalian *in vitro* systems (Brown *et al*, 1986).

Earlier *in vivo* assays have shown that mammalian transcripts cannot be spliced by yeast, using a rabbit β-globin gene on a yeast plasmid (Beggs *et al*, 1980). This is in part due to the branchpoint sequence being more variable in mammalian transcripts than in yeast. Yeast transcripts lacking this branchpoint sequence are not spliced (Langford and Gallwitz, 1983).

Although there are some differences in splicing between yeast and higher eukaryotes, yeast is an ideal model system in which to study splicing. As yeast can exist in both haploid and diploid states, it is possible to carry out detailed genetic experiments as well as biochemical assays.

1.5 *Trans*-Acting factors.

Group II introns, found in fungal and plant mitochondria, plant chloroplasts and algae, have conserved secondary structure which brings the splice sites and branchpoint together to enable splicing to occur (reviewed by Michel and Ferat, 1995). Group II introns can be spliced autocatalytically *in vitro*. Nuclear pre-mRNA splicing lacks these secondary structure elements, and an increasing body of evidence supports the theory that *trans*-acting factors must be responsible for arranging the pre-mRNA in the correct conformation for splicing (for reviews, see Wise, 1993; Weiner, 1993 and Nilsen, 1992).

Trans-acting factors involved in splicing in yeast and higher eukaryotes are proteins and small nuclear RNAs (snRNAs). Each snRNA is associated with proteins to form small nuclear ribonucleoprotein particles or snRNPs.

There are 5 small nuclear RNAs associated with the spliceosome. These are U1, U2, U4, U5 and U6. Each snRNP takes its name from the associated snRNA e.g. U1 snRNP. There are a series of core proteins which are associated with each snRNP. These are known in mammalian splicing to be B, B', D1, D2, D3, E, F and G. In addition, there are some proteins specific to particular snRNPs, such as U1A, U1C and U2B".

A multicomponent RNA-protein complex now known as the spliceosome was first isolated from HeLa cell nuclear extracts as a 60S complex from HeLa cells on a glycerol gradient (Grabowski *et al*, 1985). Denaturing gel electrophoresis of extract incubated with ^{32}P-labelled transcript under splicing conditions showed that these complexes contained precursor RNA (pre-mRNA), splicing intermediates and mature mRNA. When treated with protease or detergents, the RNA was no longer associated with the protein. The 5' and 3' consensus sequences were also shown to be involved, as a smaller complex was isolated following deletion of sequences 3' of the 5' splice site.

Another such study showed by means of a timecourse experiment that different-sized complexes were assembled sequentially during the splicing reaction (Frendeway and Keller, 1985). Using sucrose gradients, a 22S complex was the first to form, followed by a 35S complex, and finally by a 50S complex. It was also found that formation of the 50S complex required ATP. Mature mRNA was found at the top of the gradients indicating release from the complex after processing.

In yeast, a 40S complex was found associated with splicing precursor, intermediates and product. By using non-hydrolysable analogues of ATP, its assembly was also found to be ATP-dependent. When the conserved 5' splice-site and branchpoint sequences were mutated, splicing was found to be blocked (Brody and Abelson, 1985).

In yeast splicing reactions, three complexes are formed during spliceosome assembly, called complex I, II and III after their relative electrophoretic mobility in non-denaturing gels (Pikielny *et al*, 1986). An alternative naming system calling these complexes A1, A2-1, A2-2 and B respectively was proposed by Cheng and Abelson in 1987. Gel electrophoresis following time-course experiments of splicing *in vitro* have shown that the complexes are formed in the order III, I, II , and that complex II is the active spliceosome.

The first *PRP* (for precursor RNA processing) genes were isolated in a screen to identify genes involved in ribosome formation or assembly (Hartwell *et al*, 1970). It was later shown that Hartwell's mutants, *prp2-10/11* (originally named *rna2-10/11*) accumulated intron-containing RNA precursors (Lustig *et al*, 1986). Many *prp* mutants are

temperature-sensitive (ts), showing the defect at the restrictive temperature. Lin *et al* (1987) then went on to show that several of these *PRP* genes were involved in spliceosome assembly, using heat-inactivated mutant extracts. In the case of *prp2* mutant extracts, it was shown that a 40S complex did form, but these complexes contained only precursor RNA, and no intermediates or products. However splicing was shown to occur in these complexes on the addition of complementing *PRP2* splicing extracts and ATP.

Since then, around 35 *PRP* genes have been identified (Vijayraghaven *et al*, 1989; Ruby and Abelson, 1991; Maddock *et al*, 1996; Weidenhammer *et al*, 1996; Kao and Siliciano,1996). The screens carried out so far have not been exhaustive and there are thought to be more genes still unidentified. Some *PRP* genes are known to encode component parts of snRNP particles, e.g. Prp8 is a known U5 snRNP protein (Lossky *et al*, 1987).

In addition to the *PRP* genes, a number of other splicing mutants have been identified including *Brr1* to *5*, and *Ssf1* (Noble and Guthrie, 1996; Ansari and Schwer, 1995;).

In the *PRP* genes which have been sequenced so far, motifs have been identified which indicate the possible function of some of these proteins. Prp4 and Prp17 proteins has been shown to have regions of homology to the β-subunits of G proteins (Dalrymple *et al*, 1989). The homologous regions may be involved in association or dissociation of Prp4 protein with complexes during the splicing pathway.

Prp 6 and 9 proteins both have sequence motifs similar to metal-binding fingers and may be involved in RNA-binding (Legrain and Choulika, 1990). Prp22 protein is known to act in the release of mature mRNA from the spliceosome (Company *et al*, 1991) Prp26 has been shown to be an enzyme which debranches the lariat intron (Chapman and Boeke, 1991), an essential step in the degradation and recycling of splicing machinery.

Prp5 and 28 proteins are members of the DEAD-box family of putative ATP-dependent RNA helicases. Brr2 is also known as Slt22 and Yer172c and is a member of the DEXH-box subfamily.

1.7 Spliceosome assembly and splice-site selection.

Spliceosomes are known to be formed by the ordered assembly of snRNPs on to the pre-message. The first known step in spliceosome assembly is the binding of the U1 snRNP to the pre-mRNA (Mount *et al*, 1983). Experiments using RNaseT1 *in vitro* showed that the U1 snRNP was bound to the 5' splice-site. This agreed with proposed base-pairing interactions based on sequence analysis of the 5' splice site and U1 snRNA (Lerner *et al*, 1980).

It was subsequently shown that mutations in the U1 snRNA could suppress some mutations in the 5' splice-site by restoring the base-pairing interactions (Seraphin *et al*, 1988). As the restoration of base-pairing does not always suppress 5' splice-site mutations, it is thought that the actual sequence has another function as well as the base-pairing, and some proofreading mechanism is involved to prevent the aberrantly spliced transcript going through step 2 (Siliciano and Guthrie, 1988).

It has been shown in fission yeast that U1 snRNA base-pairs with both 5' and 3' splice-sites (Reich *et al*, 1992). The polypyrimidine tract at the 3' end of the intron is thought to be recognised by U1 which then binds to both the splice sites. Interaction of U1 snRNA with the 3' splice-site has been shown by making mutant 3' splice-sites, and restoring splicing by making compensatory base-changes in U1. The binding of U1 to both splice sites is essential for the subsequent binding of U2.

It was demonstrated using β-globin in a mammalian *in vitro* system that the binding of U1 snRNP is an important stage early in the formation of the spliceosome. The apparent absence of U1 snRNP in early splicing complexes is most likely explained by the use of high salt concentrations and heparin in experimental conditions, which is shown to disrupt the U1 snRNP/pre-mRNA interaction (Bindereif and Green, 1987).

The binding of the U2 snRNP to the branchpoint region of the pre-mRNA is the next stage of spliceosome assembly and the first ATP-dependent step of splicing (Parker *et al*, 1987). This was shown by using mutant yeast branchpoint sequences and looking for restoration of splicing by suppressor mutations in the 5' end of the U2 snRNA. Similar base-pairing of U2 to the branchpoint sequence in mammalian systems has also been demonstrated (Wu and Manley, 1989; Zhuang and Weiner, 1989).

Recent research provides evidence to support the model in which the base-pairing between the U2 snRNA and the conserved branchpoint sequence results in the branch adenosine residue being "bulged" out making it accessible for the transesterification reaction (Query *et al*, 1994).

Of all snRNPs, the U6 snRNP of yeast has the highest homology with its mammalian counterpart (Brow and Guthrie, 1988). It has been demonstrated to be associated with the U4 snRNP via base-pairing interactions between their respective snRNAs. Mutations in the U4 snRNA can be suppressed by making mutations in U6 snRNA which restores the base-pairing between the U4 and U6 snRNAs (Shannon and Guthrie, 1991). When extragenic suppressors of U4 snRNA mutations were isolated, it was found that dominant suppressor mutations were mutations in the U6 snRNA, and other recessive mutations were allelic with *PRP24*. Using anti-Prp24 antisera, it was shown by immunoprecipitation that the U6 snRNP was associated with Prp24 protein in wild-type cell extracts. In mutants where the U4/U6 base-pairing is disrupted, Prp24 protein co-precipitates with the U4/U6 snRNP. It is proposed that Prp24/U4/U6 is normally a transient complex in spliceosome formation and/or dissociation (Ghetti *et al*, 1995), with Prp24 being released when the U4/U6 helix is formed.

Figure 1.2. Splicing factors in the yeast pre-mRNA splicing pathway.

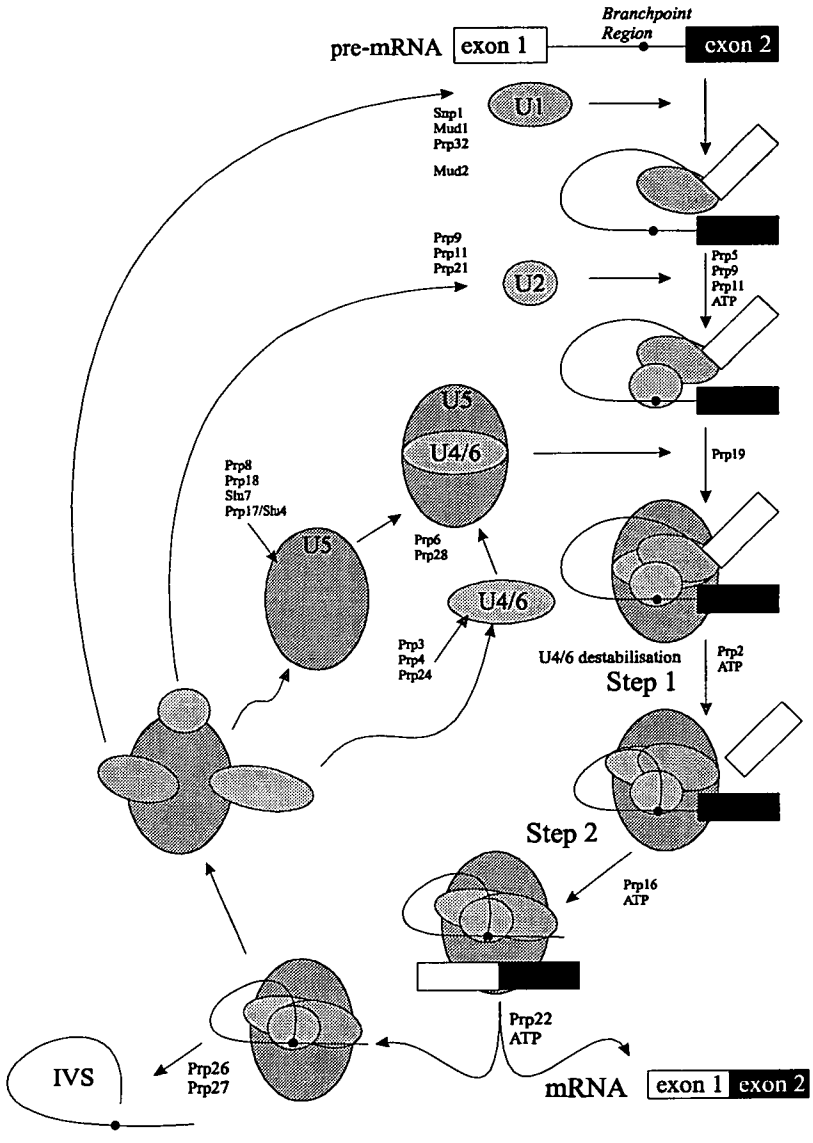


Figure 1.2: This diagram shows the proposed spliceosome assembly pathway showing associations of snRNPs with the mRNA. The proposed action points and associations for some of the Prp proteins and other *trans*-acting factors are indicated.

In mutants where the base-pairing is disrupted, Prp24 stays in association with U4 and U6. The U4/U6 complex forms an association with the U5 snRNP to make a tri-snRNP complex before joining the spliceosome (Cheng and Abelson, 1987; Lossky *et al*, 1987; Seraphin *et al*, 1991).

Prior to the first chemical step of splicing, the U4/U6 snRNA interaction is dissociated leaving U6 snRNA free to base-pair with the U2 snRNA. The helices formed between the U2 and U6 snRNAs are thought to be the basis of formation of the active site of the spliceosome (Madhani and Guthrie, 1992; McPheeters and Abelson, 1992; Madhani and Guthrie, 1994).

Newman and Norman (1991) looked for extragenic suppressors of a G1-A mutation at the 5' splice site. One such suppressor was isolated which was discovered to be the gene for U5 snRNA. The suppressor mutation was identified as a single nucleotide change in a conserved sequence encoding a single-stranded loop in the U5 secondary structure. This mutant was found to specifically suppress only mutations of G1 by cleaving the pre-mRNA 12 nucleotides upstream of the normal 5' splice-site. It is proposed that U5 normally prevents aberrantly cut mRNAs going through step 2, thus is important for splice-site fidelity.

It is now known that the conserved loop of the U5 snRNA base-pairs with nucleotides upstream of the 5' splice-site (Newman and Norman, 1992). At least in certain circumstances, it is the location of this base-pairing that determines the cleavage site. In order to allow processing of aberrantly-cut pre-mRNAs through step 2, the mutant U5 loop must also be near the 3' splice site before or during step 2. U5 snRNA has been shown to bind to two nucleotides of exon 2. U1 snRNA is also required to bind to the normal 5' splice site for the aberrant cleavage event to occur.

1.8 DEAD/H box proteins.

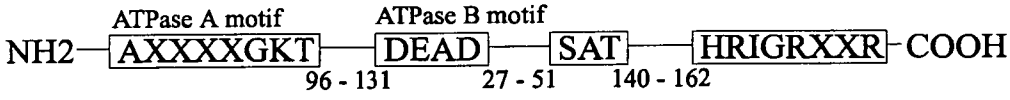
Some Prp proteins, such as Prp5 and Prp28, are members of the DEAD box family of RNA-dependent ATPases, where DEAD is a peptide sequence of Asp-Glu-Ala-Asp residues (Linder *et al*, 1989; Dalbadie-McFarland and Abelson 1990; Strauss and Guthrie, 1991). The proposed protein sequences show several motifs, all of which are present in ATP-dependent RNA helicases of which the prototype is *eIF4-A*, a translation initiation factor. (Linder *et al*, 1989). Other Prp proteins, Prp2, Prp16 and Prp22, (Chen and Lin, 1990; Burgess *et al*, 1990, Company *et al*, 1991) are members of the DEAH

subfamily where the second Asp residue is replaced by His. Some residues in the other conserved motifs in this class of proteins are also different to those in the DEAD-box proteins.

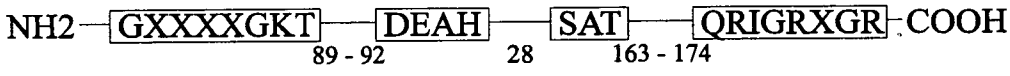
The consensus sequences of the major conserved motifs are shown below.

Figure 1.3. DEAD and DEAH-box protein consensus sequences.

DEAD Family (eg eIF4A)



DEAH Family (Prp2, Prp16 and Prp22)



The AXXXXGKT box is known as the ATPase A motif, with the DEAD box being the ATPase B motif (Linder *et al*, 1989). Mutational analysis in *eIF4-A* has shown these two conserved sequences to be important in ATP binding and hydrolysis, with GXXXXGKT having a higher rate of ATP hydrolysis than AXXXXGKT (Pause and Sonenberg, 1992).

Mutations in the DEAD and SAT motifs (to EEAD and AAA respectively) have been shown to uncouple ATPase activity from RNA helicase activity in *eIF4-A*. ATPase activity either stayed the same or increased over the wild-type in EEAD and AAA respectively. These mutants can still bind to ATP although no RNA unwinding activity was detectable (Pause and Sonenberg, 1992).

Evidence for dynamic RNA-RNA interactions in spliceosomes (reviewed in Moore *et al* 1993 and Madhani and Guthrie, 1994) strongly suggests important roles for helicases in snRNA rearrangements which occur during splicing. Proposed roles include initiating and monitoring conformational changes within the spliceosome (Wassarman and Steitz,

1991), processes which would account for some of the use of ATP during the course of the splicing reaction.

PRP2, *PRP16* and *PRP22* are members of the DEAH-box (or DEXH-box) subfamily. Their consensus sequences differ in several places from the DEAD-box prototype. These three proteins share very similar sequences in the conserved central region, with 55% identity and 75% similarity. Prp16 and Prp2 proteins both have a transient interaction with the spliceosome, Prp2 acting at step 1 and Prp16 at step 2.

The sequences related to ATP binding and/or hydrolysis may explain the need for ATP hydrolysis for splicing. The many dynamic RNA-RNA interactions which occur throughout the splicing process demonstrate the need for RNA helicase or unwindase activity in the assembly and disassembly of spliceosomes.

As mentioned in section 1.7, interactions take place between snRNAs and the pre-mRNA, such as between U1 snRNA and the 5' splice site. U2 base-pairs with the conserved branchpoint region on either side of the branch A nucleotide, which is unpaired and bulged out leaving it accessible to the first G residue of the 5' splice site (Query *et al*, 1994). There are also associations between snRNAs, for example the interaction between U4 and U6, which is dissociated to allow U6 to form helices with the U2 snRNA. There is evidence which supports the theory that U4 maintains U6 in an inactive state until it is required by the splicing machinery, whereby U4 is dissociated leaving U6 free to associate with U2. Genetic evidence suggests that the Prp16 protein interacts with the U2/U6 helix (Madhani and Guthrie, 1994b). Intramolecular helices are a key feature of the secondary structure of the snRNAs. It is conceivable that many of these interactions, which change throughout the splicing reaction, require the action of helicases. Notably, each stage of spliceosome assembly and step of splicing requires both ATP and a DEAD/H-box protein (see figure 1.2).

1.9 *PRP16*

An A to C mutation of the branch nucleotide in the *ACT1* intron has been shown to inhibit splicing (Vijayraghaven *et al*, 1986). *PRP16* was first identified through

isolation of *prp16-1* as a suppressor of this mutation which restored splicing activity (Couto *et al*, 1987). An intron-containing *ACT1-HIS4* fusion was integrated at the deleted *HIS4* locus on the genome of a diploid yeast strain. These diploids were used as a reporter strain for suppressor mutants. Integrants were tested for growth on Histidinol (Hol), the precursor of histidine on which the product of the *HIS4* gene acts. One isolate out of 521 tested was found to grow on Hol. Primer extension and Northern analysis suggests that this isolate did indeed use the C nucleotide at the branchpoint for splicing, and not a cryptic or alternative branchpoint (Vijayraghavan *et al*, 1986; Couto *et al*, 1989). Tetrad analysis showed that there was a single locus for the gene, which was called *prp16-1*.

This gene was later cloned and sequenced (Burgess *et al*, 1990). The sequence showed a 1071 amino acid open-reading frame with sequence motifs found in NTP-binding proteins. The *prp16-1* mutation was found to be a point mutation encoding a single Tyr to Asp change near the conserved ATPase A site.

Immunodepletion of splicing extracts using antibodies specific to Prp16 protein showed that spliceosome assembly and the first step of splicing did not require Prp16 (Schwer and Guthrie, 1991). This was also shown to be the case for *prp23-1*, now known to be encoded by an allele of *PRP16, prp16-2*.

The immunodepleted *PRP16* extracts could be complemented by adding a partially-purified Prp16 fraction. The dynamics of this complementation demonstrated that the spliceosome was not disassembled and reassembled on addition of Prp16 protein before splicing could take place.

PRP16 was then epitope-tagged by adding a sequence to the C terminus. This enabled purification of the Prp16 protein using a monoclonal antibody specific to the epitope tag. The purified protein was shown to complement mutant *prp16-2* extracts. RNA-dependent ATPase activity was demonstrated in the purified protein. It was then shown by immunoprecipitation that the Prp16 protein gets stuck in the spliceosome in the absence of ATP. Epitope-tagged, purified prp16-1 has subsequently been shown to have reduced ATPase activity (Schwer and Guthrie, 1992). This leads to prp16-1 getting

stuck in the spliceosome even in the presence of ATP. Pre-formed spliceosomes containing prp16-1 protein cannot be complemented by the subsequent addition of Prp16 at low temperatures. This can account for the cold-sensitive dominant-negative growth defect shown by cells heterozygous for *PRP16/prp16-1*.

The suggestion that Prp16 protein has a proofreading role in step 2 of splicing led to the proposal of a mechanism for suppression of branchpoint mutants (Burgess and Guthrie, 1993). When prp16-1 gets stuck in the spliceosome, the reduced rate of ATP hydrolysis is presumed to lower the fidelity of the proofreading mechanism, allowing the mutant branch nucleotide to be used.

Prp16 protein has been shown to induce a conformational change of the spliceosome (Schwer and Guthrie, 1992). It has also been demonstrated that Prp16 can be crosslinked to the 3' splice site of pre-mRNA in spliceosomes.

A search for suppressors of dominant-negative cold-sensitive *PRP16* mutants isolated mutants of the U6 snRNA (Madhani and Guthrie, 1994). Alleles of *PRP16* are synthetically lethal with alleles of *PRP17* and *SLU7* (Frank *et al*, 1992), which suggests that these factors interact around step two of splicing. It has been suggested that Prp16 protein may act as a molecular switch initiating a conformational change of the spliceosome which takes place at step 2 of splicing. This action may be regulated by the rate of ATP hydrolysis by Prp16 or by possible helicase activity regulating changing RNA/RNA interactions.

1.10 *PRP2*

The *PRP2* gene was first cloned and sequenced in this lab (Lee *et al*, 1984). The gene was cloned by transforming a temperature-sensitive (ts) *prp2-1* strain with a YEP24-based plasmid library, and selecting for transformants at the non-permissive temperature, 36°C. One transformant was isolated and the plasmid extracted. This plasmid was found on transformation to rescue the ts phenotype of four different *prp2-1*

strains. *PRP2* was shown to be a single gene with a 3.2kb *Eco*RI-*Bam*HI fragment essential for the rescue of the ts phenotype.

Figure 1.4. Map of *PRP2* showing unique restriction sites.

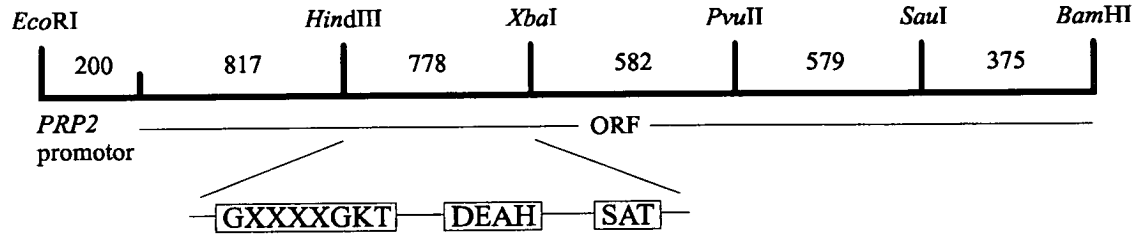


Figure 1.4 Restriction map of *PRP2* including the 200bp promoter region and the 3kb coding region. Restriction fragment sizes are shown in bp. Conserved protein motifs encoded by the *Hin*dIII-*Xba*I fragment are indicated below.

Immunoprecipitation using Prp2-specific antisera has shown Prp2 protein to be associated with the spliceosome before and during step 1 of splicing (King and Beggs, 1990). This association was found not to happen in the presence of a mutant branchpoint or 5' splice site, or in the absence of ATP. When *prp2-1* mutant extracts were heat-inactivated, spliceosomes could no longer be precipitated by anti-Prp2 antisera, suggesting that heat-inactivated prp2-1 protein is not associated with the spliceosome.

As mentioned above, Prp2 is a member of the DEAH subfamily of DEAD-box proteins (Company *et al*, 1991). It shares extensive sequence homology with Prp16 and Prp22 over their central regions, with 55% identity and 75% similarity, although each protein has differing NH_2- and COOH- termini. Prp16 and Prp2 proteins both have a transient interaction with the spliceosome, Prp2 acting at step 1 and Prp16 at step 2. Prp22 protein has recently been shown to have RNA helicase activity *in vitro* (Wagner *et al*, 1996). This is the first example of helicase activity known in this group of proteins.

The presence of sequence motifs common to both Prp2 and Prp16 has lead to speculation that these proteins carry out a similar role in the splicing pathway, with different specificities encoded by the different 5' and 3' sequences of these genes. As a proofreading role has been suggested for *PRP16*, it is exciting to speculate that *PRP2*

may have a similar role at an earlier step of splicing. Genetic evidence suggests that Prp2 protein interacts with at least one other protein factor, Spp2, during step one of splicing (Last *et al*, 1987; Roy *et al*, 1995).

The interaction between Prp2 protein and the spliceosome is of a transient nature. In order to facilitate the study of this interaction, dominant negative mutants have been generated which enter the spliceosome and form a stable association preventing splicing from taking place (Plumpton *et al*, 1994; E. Flinn, 1994). Random dominant negative mutants were generated (Plumpton *et al*, 1994) which, when sequenced, all contained a mutation which resulted in a serine to leucine change in the SAT box, which in eIF4A is required for RNA unwinding activity. This mutant, $PRP2^{dn1}$, inhibited growth when overexpressed from a conditional promoter in a wild-type strain. *In vitro* analysis of this mutant showed inhibition of splicing and the stalling of splicing complexes. Immunoprecipitation experiments with anti-Prp2 antibodies demonstrated that the normally transient interaction between Prp2 and the spliceosome was to some extent stabilised by the dominant negative mutant, which, unlike the wild-type Prp2 protein, remained associated with the spliceosome in the presence of ATP. Assays carried out with purified $Prp2^{dn1}$ protein demonstrated that $Prp2^{dn1}$ had around 40% of the RNA-stimulated ATPase activity of the wild-type protein.

UV crosslinking experiments were carried out in an *in vitro* system using $Prp2^{dn1}$ (Teigelkamp *et al*, 1994). These experiments showed that the Prp2 protein directly contacted the pre-mRNA. This reaction was independent of an intact 3' splice site, but was shown to require a pre-mRNA capable of formation into an intact spliceosome. Experiments using an ATP-depleted system and purified wild-type Prp2 protein showed that the results obtained for $Prp2^{dn1}$ were also true for wild-type Prp2 protein.

Subsequent dominant negative mutants of *PRP2* were generated by site directed mutagenesis of the GKT-box (E. Flinn, 1994). Mutations resulting in GKT to GKN ($Prp2^{GKN}$) and AKT ($Prp2^{AKT}$) substitutions were made and found to be dominant negative. The $Prp2^{GKN}$ mutant was found to be similar to $Prp2^{dn1}$, forming a stable association with the spliceosome. In contrast to $Prp2^{dn1}$, ATPase activity in both the $Prp2^{GKN}$ and $Prp2^{AKT}$ mutants was found to be negligible. The dominant negative

phenotype of the Prp2^{AKT} mutant was also different, exhibiting dominance upon overexpression only at 30°C and under. A stable association between Prp2^{AKT} and the spliceosome has never been demonstrated. However, Prp2^{AKT} has been demonstrated to bind to poly(U) *in vitro* (M. McGarvey, unpublished results).

1.11 RAS

One dominant negative mutation of *PRP2* was inspired by a mutation made in *RAS* which gave a dominant negative phenotype. Both mammalian H-ras and yeast Ras1 and Ras2 proteins contain similar sequences to those conserved between Prp2, Prp16 and Prp22. Mutagenesis resulted in a GKT to AKT change (Powers *et al*, 1989).

Figure 1.5. The GTPase cycle.

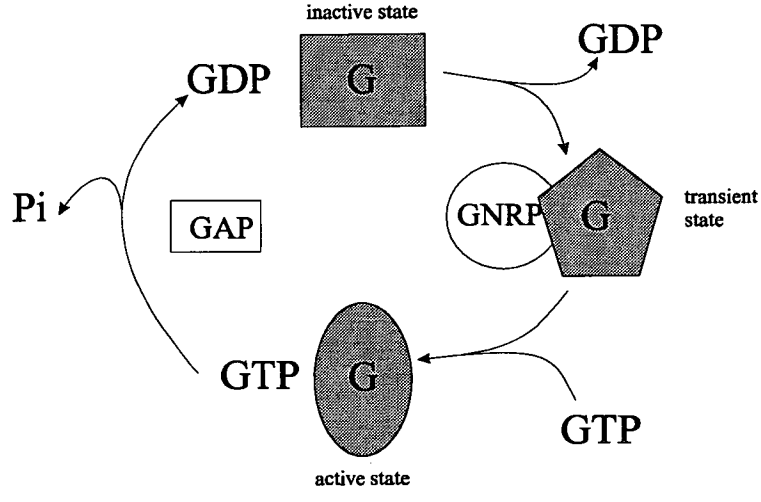


Figure 1.5. G represents the GTPase protein, GAP is the GTPase activating protein, and GNRP is the guanine nucleotide release protein. The GNRP serves to stabilise the transient nucleotide-free form of the GTPase.

This mutation is thought to confer dominant negative activity on Ras by stabilising the interaction between Ras2 and the nucleotide exchange factor, Cdc25. The Cdc25 protein acts upstream of Ras2 which exchanges GDP for GTP thus activiating Ras (Broek *et al*, 1987).

The dominant mutation in Ras2 is believed to stabilise the normally transient interaction between Ras2 and Cdc25 which occurs around the time of exchange of GDP for GTP by Ras2. This stabilisation results in free Cdc25 protein being depleted. It has been suggested that a similar mutation in *PRP2*, $PRP2^{AKT}$, results in the stabilisation of an interaction between Prp2 protein and a possible nucleotide exchange factor (E. Flinn, 1994).

1.12 This thesis.

The aim of this thesis was to investigate the role in splicing of conserved sequences in the Prp2 protein by mutagenesis.

The first sequence to be investigated was the zinc finger-like motif. A strategy was developed to mutagenise key amino acid residues involved in zinc finger function.

Deletion analysis of three dominant-negative alleles of *PRP2* was carried out in order to map RNA-binding activity. This led to a suggestion that the Prp2 protein may interact with a factor involved in splicing complex formation. An attempt was made to investigate this hypothesis.

Finally, the similarity between *PRP2* and *PRP16* led to the investigation of a possible proofreading role for Prp2 protein in splicing.

Chapter two.

Materials and methods.

2.1 Materials.

2.1.1 Suppliers of laboratory reagents.

Standard laboratory reagents were purchased from Sigma, Fisons and BDH.

Restriction endonucleases and nucleic acid modifying enzymes were purchased from Boehringer, New England Biolabs, Gibco BRL and Pharmacia.

Deoxyribonucleotides and ribonucleotides were purchased from Pharmacia and Sigma.

Radiochemicals were purchased from Amersham International.

Acrylamide was purchased from Appligene and Scotlab.

Media reagents were purchased from Difco, Oxoid, Sigma and Fisons.

2.1.2 Growth Media

Unless otherwise stated quantities are for 1 litre volume. Solutions were autoclaved (section 2.2.2) and stored at room temperature. Antibiotics and amino acids were stored as sterile concentrated stocks and were added to media immediately prior to use. For plates, Oxoid No.1 agar was added to 2% (w/v) before autoclaving.

2.1.2.1 *E.coli* media.

Luria-Bertani medium (LB):

10g peptone, 5g yeast extract, 10g NaCl, adjusted to pH7.2 with NaOH.

0.1g/l ampicillin was added as necessary (LB-amp).

M9 medium:

6g Na_2HPO4, 3g KH_2PO4, 0.5g NaCl, 1g KH_4Cl. 0.25g $MgSO_4.7H_2O$, 2g glucose.

20mg amino acids and other nutritional requirements were added after autoclaving.

2XYT:

10g peptone, 10g yeast extract, 5g NaCl.

BBL Bottom plates:

10g trypticase, 5g NaCl, 10g agar.

BBL Top:

10g trypticase, 5g NaCl, 6.5g agar.

2.1.2.2 Yeast Media.

YPDA:

10g yeast extract, 10g peptone, 20g glucose, 20mg adenine sulphate.

YMGlu:

6.7g yeast nitrogen base without amino acids, 20g glucose, supplemented with 20mg amino acids as required.

YMGal/Raf:

6.7g yeast nitrogen base without amino acids, 20g galactose, 20g raffinose, supplemented with 20mg amino acids as required.

YMGluCas, YMGal/RafCas:

YMGlu or YMGal/Raf plus 10g vitamin assay casaminoacids. 20mg adenine, tryptophan and uracil were added after autoclaving as required.

Pre-sporulation:

8g yeast extract, 3g tryptone, 100g glucose.

Sporulation:

10g potassium acetate, 1g yeast extract, 0.5g glucose, 2mg required amino acid.

YMGlyLacCas:

6.7g yeast nitrogen base without amino acids and 10g vitamin assay casaminoacids in 800ml H_2O. After autoclaving, 100ml sterile 20% lactic acid pH5.7 and 100ml sterile 20% glycerol were added. For galactose induction, galactose was added to 2% (w/v).

5-FOA:

6.7g yeast nitrogen base without amino acids, 20g glucose, 2g vitamin assay casaminoacids, 50mg uracil, 2g agar in 500ml H_2O, autoclaved. 1g 5-fluoroorotic acid dissolved in 500ml H_2O, filter sterilised and added to the autoclaved mixture before pouring into Petri dishes.

Synthetic Complete (SC):

20g Glucose or Galactose plus the following:

Supplement	Final concentration (mg/l)	Stock per 100ml	Amount of stock per litre
Adenine sulphate	20	200mg [a]	10
Uracil	20	200mg [a]	10
L-Tryptophan	20	1g	2
L-Histidine-HCl	20	1g	2
L-Arginine-HCl	20	1g	2
L-Methionine	20	1g	2
L-Tyrosine	30	200mg	15
L-Leucine	30	1g	3
L-Isoleucine	30	1g	3
L-Lysine-HCl	30	1g	3
L-Phenylalanine	50	1g [a]	5
L-Glutamic acid	100	1g [a]	10
L-Aspartic acid	100	1g [a,b]	10
L-Valine	150	3g	5

L-Threonine	200	4g [a,b]	5
L-Serine	400	8g	5

a- stored at room temperature (other stocks stored at 4°C)

b- added to media after autoclaving.

2.1.3 Bacterial strains.

Table 2.1 *E.coli* strains

Strain	Genotype	Description	Reference/origin
NM522	Δ(*lac-proAB*), Δ(*hsdMS-mcrB*)5, *supE, thi-1*, F'[*lacI*^{q}, *lacZΔ M15, proAB*]	Strain for propagating plasmids and phagemids	Gough and Murray, 1983
NM547	*dam*$^{-}$, *ara, B1, Δ(lac-pro), rpsL, Sm*^{R}	Methylation minus strain for propagating plasmids with methylation-sensitive restriction sites.	N. Murray
JDB1	*dam*$^{-}$, *ara, B1, Δ(lac-pro), rpsL, Sm*^{R} F'[*lacI*^{Q}, *lacZΔ M15, proAB*]	Methylation minus strain for propagating plasmids and phage with methylation-sensitive restriction sites.	J. Beggs, this lab.
BMH71-18*mut*L	*Δ(lac,pro)*, *sup*E, *thi*$^{-}$, *mut*L, *F' (proAB, lacI, lacZΔM15)*.	Repair-deficient strain for site-directed mutagenesis.	Kramer *et al*, 1984
BW313	HfrKL16 PO/45 *lysA* (61-62), *dut1*, *ung1*, *thi-1*, *recA*, *SpoT1*	Repair-deficient strain for site-directed mutagenesis.	Amersham International

2.1.4 Yeast strains.

Table 2.2 Yeast strains.

Strain	Genotype	Description	Reference/origin
S150-2B	**a**, *ura3-52, leu2-3,-112, trp1-289 GAL+.*	general strain for transformation and splicing extracts	Wellcome
BJ2412	**a**, *gal2, leu2, pep3-4, prb1-11,prc-407, trp1, ura3-52.*	protease deficient strain for splicing extracts	E. Jones (Pittsburgh)
DJy85	**a/α**, *prp2-1/prp2-1, ura3/ura3, ade1/ADE1, ade2/ade2, trp1/TRP1, his3/HIS3, tyr1/TYR1, lys2-801/LYS2, can1/CAN1.*	diploid homozygous for the temperature-sensitive *prp2-1* allele	D. Jamieson (this lab.)
DJy36	**a**, *ura3-2/52, ade2, prp2-1.*	haploid temperature-sensitive *prp2-1* strain	D. Jamieson (this lab.)
EFy1	**a/α**, *ura3-52/ura3-52, his3-Δ 200/his3Δ1, ade2-101oc/ADE2, lys2-801/LYS2, trp1-Δ1/trp1-289, LEU2/leu2-3,112, PRP2/PRP2::HIS3.*	diploid with one genomic copy of *PRP2* disrupted by *HIS3.*	E.Flinn (PhD thesis, this lab.)
51	**a**, *lys2, his3, ura3, trp1, leu2, ade, GAL, cup1.*	*cup1* strain for β-galacosidase assays	Guthrie lab. (UCSF)
46	**α**, *lys2, his3, ura3, trp1, leu2, ade, GAL, cup1.*	*cup1* strain for β-galacosidase assays	Guthrie lab. (UCSF)

2.1.5 DNA constructs.

Table 2.3 DNA constructs.

Construct	Description	Reference/origin
pY2000	2μ *PRP2* construct	Lee *et al*, (1984)

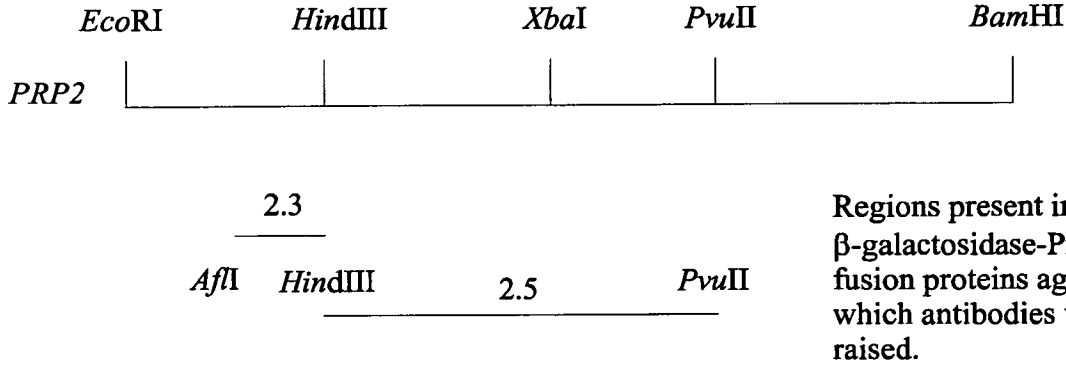
pBM125	*ARS, CEN, URA3* vector containing the *GAL1* promoter on a 0.8kb *Eco*RI-*Bam*HI fragment	Johnston and Davies, (1984)
pBMPRP2	GAL inducible *PRP2*	King and Beggs, 1990
pBMPRP2^{His}	Polyhistidine tagged GAL *PRP2*	Plumpton *et al*, (1994)
pBMPRP2^{LATHis}	Polyhistidine tagged GAL *PRP2^{LATHis}*	Plumpton *et al*, (1994)
pBMPRP2^{AKTHis}	Polyhistidine tagged GAL *PRP2^{AKTHis}*	E.Flinn (this lab.)
pEF2	*PRP2::URA3*	E.Flinn (this lab.)
M13P2C2	M13 phage *PRP2/C2*	E. Whittaker, J. Morran (this lab.)
pFL39	2μ *TRP1* vector	Bonneaud *et al*, (1991)
pRS315		Sikorski and Heiter, (1989)
pSB1	2μ *prp16-1*	Burgess and Guthrie, (1993)
pBS36	*ACT1/CUP1* mutant fusion	"
pSB47	*ACT1/CUP1* mutant fusion	"
pSB48	*ACT1/CUP1* mutant fusion	"
pSB49	*ACT1/CUP1* mutant fusion	"
pSB50	*ACT1/CUP1* mutant fusion	"
pG100	*ACT1/CUP1* mutant fusion	Lesser and Guthrie, (1992)
pG14	*ACT1/CUP1* wild type fusion	"
pG24	*ACT1/CUP1* wild type fusion	"
pG1PRP16epit	2μ *PRP16* with *PG1* promoter	"
pYAHB2/A1	*ACT1/lacZ* mutant fusion	Vijayraghaven *et al*, (1986)
pYAHB2/WT	*ACT1/lacZ* wild type fusion	"
pTZ18	*E.coli* phagemid	Pharmacia
pTZ19	*E.coli* phagemid	Pharmacia
pT7/rp28s	Template for *in vitro* transcription of *rp28*	M. Lossky (this lab.)

2.1.6 Antisera.

Table 2.4 Antisera.

Antiserum	Antigen	Origin
Anti-54	Fusion 2.3 protein. (see figure 2.1).	King and Beggs (1990)
Anti-55	Fusion 2.3 protein.	D.King (this lab)
Anti-140	Fusion 2.5 protein (see figure 2.1).	D.King (this lab)

Figure 2.1. Anti-Prp2 antibodies used in this thesis.



Regions present in β-galactosidase-Prp2 fusion proteins against which antibodies were raised.

2.1.7 Oligodeoxyribonucleotides.

All oligodeoxyribonucleotides were synthesised by the Oswel DNA Service (Edinburgh University).

Table 2.5 Oligodeoxyribonucleotides

Oligo	Description	Sequence (5'-3')
036A	Universal Primer	CCCAGTCACGACGTT
413G	Reverse primer	AACGTCGTGACTGGG
163H	His-Leu in MBF of Prp2 by PCR	TACAGCTGCTACGCTCGAAC
164H	Cys4-Ser in MBF of Prp2 by PCR	TACAGCTGCTACGCACGAACA ATCTCAAGG

165H	Cys5-Ser in MBF of Prp2 by PCR	TACAGCTGCTACGCACGAACA ATGTCAAGGTGTACTAGAAGA GTCTTTGACC
168H	*PRP2* PCR oligo in reverse orientation to 163-165H	GCGGATCCAAACAATCTC
029A	*PRP2* primer at nt1610	GATTATAACTCCTATA
028A	*PRP2* primer at nt1196	TCTTACAGATTCCAAA
131K	*PRP2* primer at nt2339	CCAATGGAGAAACTC
027A	*PRP2* primer at nt1970	TACGGTGTTATTATTA
448K	Cys2-Ser in MBF of Prp2	TTTCCTTCTGAACCTGA
026A	Cys2-Tyr in MBF of Prp2	TTTCCTTATGAACCTGA
882F	His-Cys in MBF of Prp2	GCTGCTACGTGCGAACAATGT
A171	T-Val in GKT box of Prp2	CCAGAACCGACTTCACCC
883F	Cys1-Tyr in MBF of Prp2 (replaces oligo 025A)	ATGATGTACGAGTTTCC
10K	Makes Tyr-Asp change in Prp2 (homologous to *prp16-1* mutation)	GTTACCCCAAGATTTGGTAG
132K	*PRP2* primer at nt2533	GATGCTTCATCAGCGGG
304L	*PRP2* primer at nt	GGGCAAGAAGAAATTG
JB1	Self anneals to form blunt ended linker with diagnostic *Cla*I site.	CTAATCGATTAG
446K/447K	Hybridise to form *Xba*I-*Sac*I-*Pvu*II linker	CTGCCGAGCTCGCT/CTAGAGC GAGCTCGGCAG
449J/450J	Hybridise to form *Pvu*II-*Sac*I-*Sau*I linker	CTGCGAGCTCGCC/TTAGGCGA GCTCGCAG
484K/485K	Hybridise to form *Hin*dIII-*Sac*I-*Xba*I linker	AGCTTCGGAGCTCGCT/CTAGA GCGAGCTCCGA
166H/167H	Hybridise to form *Pvu*II-*Sac*I-*Bam*HI linker	GATCCGGAGCTCGCAG/CTGCG AGCTCCG

2.1.8 Size markers.

Size markers for agarose gel electophoresis were Lambda DNA (Gibco BRL) digested with *Hin*dIII restriction endonuclease, and 1kb ladder (Gibco BRL).

High molecular weight markers from Sigma (SDS-6H) were used for SDS-PAGE of proteins.

2.1.9 Buffers.

1M $NaPO_4$	1M NaH_2PO_4 and 1M Na_2HPO_4 at 3:2 (v/v) to pH7.
10X PBS	15mM NaH_2PO_4, 80mM Na_2HPO_4 , 1.5M NaCl pH7.2
10X TAE	0.4m Tris-acetate pH7.5, 20mM EDTA.
10X TB	0.9M Tris-borate pH8.3.
10X TBE	0.9M Tris-borate pH8.3, 20mM EDTA.
10X TBS	0.5M Tris-HCl, 1.5M NaCl pH7.5.
10X TE	10mM Tris-HCl, 1mM EDTA pH8.0.
20X SSC	3M NaCl, 0.3M tri-sodium citrate pH7.0.

2.2 General Methods.

2.2.1 General guidelines.

Unless otherwise stated all procedures were carried out at room temperature. For small scale procedures sterile 1.5ml polypropylene microcentrifuge tubes (Treff) were used, larger scale procedures being carried out in 15ml and 30ml sterile Corex tubes, 10ml Sterilin tubes and 50ml Greiner tubes. 1 to 4l cell cultures were harvested in 250ml or 500ml Nalgene polycarbonate or polyallomer centrifuge bottles.

Centrifugation of volumes greater than 50ml was carried out in a Sorvall Super Speed centrifuge using either a GS-A or GS-3 rotor at 5,000 rpm. Volumes between 1.5 and 50ml were centrifuged in an SS34 rotor at speeds between 10,000 and 17,000 rpm or in an IEC Centra-4X bench top centrifuge at 4,000 rpm. High speed centrifugation was performed in a Sorvall OTD ultracentrifuge. Volumes of 1.5ml or less were centrifuged in an Eppendorf centrifuge at 14,000 rpm.

2.2.2 Sterilisation procedures.

When necessary, plastics such as microcentrifuge tubes and pipette tips, and solutions were sterilised by autoclaving for 20 minutes at 120°C and 15 psi. Solutions not suitable for autoclaving and small volumes were sterilised by filtration through a disposable Acrodisc syringe filter (0.45μm pore size, Gelman Sciences).

Sterile and RNase-free glassware was dry-sterilised by baking at 250°C for 16h.

2.2.3 Deionisation of solutions.

Formamide and acrylamide solutions were deionised by mixing with 0.1-0.2 volumes of analytical grade mixed bed resin (20-50 mesh, Bio-Rad) for 30 minutes. The resin was removed by filtration through Whatman No.1 filter paper. Acrylamide was stored at 4°C and formamide at -20°C.

2.2.4 Photography.

Photographs of agarose gels stained with 1μg/ml ethidium bromide were taken using a UVP electronic imaging system during visualisation of the nucleic acid by short wave ultraviolet light (254nm) from a UVP transilluminator. Photographs of yeast growing on Petri dishes were taken using the same system, using a white lightbox instead of the transilluminator.

2.2.5 Autoradiography.

[^{32}P]-labelled nucleic acids were detected by exposing pre-flashed X-ray film to the subject in a light proof cassette with a calcium-tungstate intensifying screen at -70°C.

[^{35}S]-labelled DNA was detected by exposure of X-ray film to dried gels at room temperature.

2.3 MICROBIOLOGICAL METHODS.

2.3.1 Propagation and storage of bacterial strains.

Growth and storage of *E.coli* were carried out as in Sambrook *et al* (1989).

2.3.2 Preparation of competent *E. coli.*

Based on the method of Hanahan (1983).

1) 100ml of LB was inoculated with 1ml of a stationary phase culture and grown to OD_{600} 0.3-0.7.

2) The culture was chilled on ice before cells were harvested in a benchtop centrifuge. The cell pellet was resuspended in 50ml ice-cold 75mM $CaCl_2$ and incubated on ice for a minimum of 20 min.

3) Cells were pelleted again and resuspended in 10ml ice-cold 75mM $CaCl_2$. These were either used immediately or stored at 4°C for up to 48h.

2.3.3 Transformation of competent *E. coli* with plasmid DNA.

1) Competent cells were aliquoted into 200µl volumes, DNA added (about 50-100ng plasmid or a portion of a ligation) and incubated on ice for a minimum of 20 min.

2) The cells and DNA were heat shocked at 42°C for 2-5 min.

3) 1ml of LB was added, and the cells incubated at 37°C for 1h to allow expression of the antibiotic resistance gene.

4) The cells were pelleted by spinning in a microcentrifuge and most of the supernatant poured off.

5) The cells were resuspended in the remaining supernatant and spread onto LB-amp plates which were incubated inverted at 37°C overnight.

2.3.4 Transfection of competent *E. coli* with phage DNA.

1) DNA (as for transformation) was added to 200µl aliquots of competent *E.coli* in sterile 3ml glass test tubes and incubated on ice for a minimum of 20 min.

2) The cells were heat shocked at 42°C for 2-5 min. and returned to the bench.

3) 200µl stationary cells and 3ml cooled (no more than 42°C) molten BBL top agar was added and mixed by inversion. The stationary cells provide a lawn in which plaques may form.

4) The contents were poured onto a BBL bottom plate.

5) When set, the plate was inverted and incubated at 37°C overnight.

2.3.5 Propagation and storage of yeast.

Yeast cells were grown and stored as described in Rose *et al* (1990).

2.3.6 Sporulation and spore recovery.

Diploid strains to be sporulated were grown first on presporulation plates for one or two days and then on sporulation plates for 3-4 days at 25°C or 30°C.

Asci were resuspended in spore buffer (1.2M sorbitol, 10mM EDTA, 100mM citric acid). 20µl β-glucuronidase/arylsulphatase (Boehringer) was added to digest the ascus walls. After incubation at room temperature for 20 min*, dissection of asci was carried out using a Singer MSM micromanipulator (Singer Instrument Co.), the four haploid spores being placed separately in a row on a plate of an appropriate medium.

The plate was incubated at 25°C or 30°C until the spores grew into colonies (around 3-6 days).

*The digested asci can be stored at 4°C overnight at this stage.

2.3.7 Transformation of yeast.

(Geitz *et al*, 1992)

1) A 100ml yeast culture was grown in complete medium to OD_{600} 0.4-0.7.

2) Cells were harvested, washed with 20ml sterile 1X TE/1X lithium acetate, and resuspended in 250µl 1X TE/1X lithium acetate.

3) 1-10µg DNA to be transformed, 5µl salmon sperm DNA (100mg/ml), and 300µl 50% PEG was added to 50µl aliquots of cells and incubated with agitation at 25°C or 30°C for 30 min.

4) Cells were heat shocked at 42°C for 15 min, then spun down.

5) The supernatant was removed and the cells resuspended in 50µl sterile dH_2O before being spread on selective medium and incubated at 25°C or 30°C for 2-5 days until colonies appeared.

2.4 Nucleic Acid Methods.

2.4.1 General methods.

Many standard DNA and RNA manipulations were carried out either according to Sambrook *et al* (1989) or according to manufacturers instructions. These included quantitation of nucleic acids by spectrophotometry, restriction endonuclease digestion of DNA, dephosphorylation of DNA, agarose gel electrophoresis of DNA and RNA, purification of DNA fragments from agarose gels (Qiaex, Diagen Inc.), and ligation of DNA fragments. All DNA was stored in sterile ddH_2O at 4°C, and RNA stored in ddH_2O at -20°C. Main stocks of oligonucleotides were stored at -70°C with aliquots at -20°C.

2.4.2 DNA preparations.

2.4.2.1 Small scale plasmid preparation.

Based on the method of Birnboim and Doly (1979).

1) 5ml LB-amp was inoculated with a single bacterial colony and incubated at 37°C with shaking for 5h or overnight.

2) 1.5ml of the culture was harvested in a microcentrifuge tube by spinning for 10min.

3) The liquid was discarded and the cell pellet resuspended in 100µl solution A and incubated at room temperature for 5min.

4) 200µl solution B was added and the lysed cells mixed by inversion.

5) 150µl solution C was added, the contents mixed thoroughly by inversion and placed on ice for 5min.

6) Cell debris was removed by spinning for 15min and the supernatant transferred to a fresh tube.

7) The supernatant was extracted once with an equal volume of phenol, once with phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1).

8) 2-3 volumes of absolute ethanol were added to the aqueous phase, mixed well and spun at 4°C for 20min.

9) The pellet was washed once with 70% (v/v) ethanol, dried in a vacuum desiccator and dissolved in 30µl dH_2O.

Buffers.

A: 50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA.

B: 0.2M NaOH, 1% (w/v) SDS.

C: 3M sodium acetate pH5.2.

2.4.2.2 Large scale plasmid preparation.

1) 50ml LB-amp was inoculated with 10µl of a stationary culture and incubated overnight with agitation at 37°C.

2) The cells were harvested by spinning in a benchtop centrifuge at full speed for 20min.

3) The cells were resuspended in 2ml solution A and incubated on ice for 5min.

4) 4ml solution B were added, mixed by inversion and incubated on ice for 5min.

5) 3ml solution C were added, mixed well by inversion and incubated on ice for 5min.

6) This was transferred to a 50ml polycarbonate tube and spun for 10min at 10krpm at 4 °C.

7) The supernatant was transferred to a 30ml Corex tube and 16ml of ethanol added.

8) This was incubated at -20°C for 30min before spinning down the DNA at 4°C at 10krpm for 10min.

9) The pellet was resuspended in 2ml buffer D and extracted once with 2ml phenol/chloroform. The aqueous phase was removed and the phenol phase back extracted with another 2ml of buffer D.

10) The two aqueous phases were pooled in a 15ml Corex tube and 8ml ethanol added before incubating at -20°C for 30min.

11) The DNA was pelleted at 10K rpm and the pellet resuspended in 400µl TE.

12) RNase A was added to a concentration of 0.2µg/µl and incubated at 37°C for 45min.

13) RNase treated DNA was extracted once with phenol, once with phenol/chloroform and once with chloroform before 0.1 volumes 3M sodium acetate and 2 volumes ethanol were added to precipitate the DNA.

14) After incubation at 4°C for 30min and spinning at 4°C for 30min, the pellet was washed with 70% (v/v) ethanol, dried under vacuum and dissolved in 200µl ddH_2O.

This method routinely yielded 100-200µg DNA.

Buffers.

Solutions A, B and C as section 2.4.2.1.

Solution D: 0.1M sodium acetate pH 5.2, 1mM EDTA, 0.1% (w/v) SDS, 40mM Tris-HCl pH 8.0.

2.4.2.3 Preparation of replicative form (RF) DNA from M13

As above except:

Harvested cells were washed with 20ml STE after step 2.

STE: 0.1M NaCl, 10mM Tris pH8.0, 1mM EDTA pH8.0

2.4.2.4 Preparation of single stranded DNA from M13 phage.

1) A 1.5ml culture of NM522 was grown in 2XYT to OD_{600} 0.3 at 37°C.

2) A fresh plaque was picked into the culture of NM522 and grown for 5-6h at 37°C with vigourous shaking.

3) The cells were pelleted by spinning for 5min.

4) 1ml of the supernatant was transferred to a fresh tube.

5) 150µl PEG/NaCl was added and incubated at room temperature for 20min.

6) The phage were pelleted by spinning for 10min. (If no pellet was present, the tube was discarded.)

7) The PEG/NaCl was removed and the pellet resuspended in 100µl $T_{10}E_{0.1}$.

8) This was extracted with phenol and precipitated with ethanol before being dried and resuspended in 25µl $T_{10}E_{0.1}$.

Buffers.

PEG/NaCl: 20% (w/v) PEG 6000, 4M NaCl.

$T_{10}E_{0.1}$: 10mM Tris pH7.5, 0.1mM EDTA.

2.4.2.5 Preparation of single stranded DNA from pTZ phagemids.

1) 1.5ml 2XYT was inoculated with 30µl of a stationary culture of *E. coli* transformed with the recombinant pTZ, and grown at 37°C with vigorous shaking for 30min.

2) 4.5µl M13KO7 helper phage were added and the culture grown for a further 5-6h at 37°C with vigorous shaking.

3) The cells were pelleted and the supernatant transferred to a fresh tube.

4) 150µl PEG/NaCl was added and incubated at room temperature for 20min.

5) Phage were pelleted by spinning for 10min. The supernatant was removed and the tubes spun again and residual supernatant removed.

6) The phage pellet was resuspended in 100µl $T_{10}E_{0.1}$ and extracted once with phenol/chloroform.

7) The DNA was precipitated by adding 0.1 volume 3M sodium acetate pH 5.3 and 2 volumes ethanol.

8) After precipitation the pellet was dried under vacuum and resuspended in 25µl $T_{10}E_{0.1}$.

Buffers: as section 2.4.2.3

(M13KO7 was prepared as recommended by the supplier (Pharmacia))

2.4.3 DNA sequencing.

All sequencing reactions were carried out using the Sequenase version 2.0 kit (USB/Amersham) according to the instructions provided. Templates for the reactions were single stranded preparations of recombinant pTZ phagemids (7µl of preparation) or M13 phage (4µl of preparation). Products of sequencing reactions were run on 6% (w/v) acrylamide/7M urea denaturing gels in 1XTB buffer, fixed in 10% (v/v) methanol 10% (v/v) acetic acid for 20min and dried on a vacuum gel drier.

2.4.4 Site-directed mutagenesis.

2.4.4.1 Site-directed mutagenesis based on the method of Kunkel *et al* (1987).

1) Generation of uracil-containing template DNA:

Double stranded recombinant pTZ DNA was transformed into the *dut*$^-$, *ung*$^-$ *E.coli* strain BW313, and a single transformant grown overnight. This culture was used to prepare single stranded template DNA as described in section 2.4.2.4. BW313 lacks the dUTPase responsible for metabolising dUTP so DNA synthesis proceeds with dUTP.

2) Kinasing the synthetic oligodeoxyribonucleotide:

The oligodeoxyribonucleotide carrying the desired mutation (see table 2.5) was phosphorylated as follows: 200pmol oligodeoxyribonucleotide, 3µl 1M Tris-HCl pH8.0, 1.5µl 0.1M DTT, 1.0µl 0.2M $MgCl_2$, 1.0µl 10mM ATP and 5 units T4 polynucleotide kinase, combined in a 30µl final volume. This was incubated at 37°C for 1h before inactivating the enzyme at 65°C for 30min.

3) Annealing the synthetic oligodeoxyribonucleotide to template DNA:

0.5pmol template DNA, 5pmol kinased oligo and 2µl annealing buffer were combined in a final volume of 10µl. The mixture was heated to 65°C for 2min and cooled slowly to room temperature.

4) Polymerisation and elongation reaction:

10µl annealed oligo/template, 1µl buffer B, 4µl deoxyribonucleotide mix, 1µl 10mM ATP, 2 units T4 DNA ligase and 2 units Klenow fragment were combined in 20µl final volume and incubated overnight at 15°C .

5) Introduction into wild type *E. coli*:

The double stranded products of the reaction were transformed into a repair-proficient (*DUT, UNG*) strain, NM522. This results in U-containing DNA being digested leaving the mutated T-containing clones. Mutant clones were identified by sequencing single stranded DNA from single transformants. This method reliably yielded at least 66% mutagenesis.

Buffers.

5X annealing buffer: 200mM Tris-HCl pH7.5, 100mM $MgCl_2$, 250mM NaCl.

Buffer B:

0.2M Tris-HCl pH 7.5, 0.1M $MgCl_2$, 0.1M DTT.

Deoxyribonucleotide mix: 2mM each of dATP, dCTP, dGTP and dTTP.

2.4.4.2 Site-directed mutagenesis by PCR.

1) 0.3-6µg each oligo and 50ng template DNA was assembled in a 100µl reaction with 10µl 10X buffer (supplied with the polymerase) and 10µl stock solution nucleotides. The lid of the tube was pierced and the contents overlaid with mineral oil.

2) The reaction was heated to 91°C for 5min and cooled to room temperature.

3) 2.5 units of enzyme were added and the reaction put through 25 cycles as follows:

1min 92°C,

1min lowest Tm of oligos -5°C,

2min 72°C.

PCR reactions were stored at -20°C.

2.4.5 Extraction of RNA from yeast.

2.4.5.1 Small scale.

1) 10ml yeast cells were taken from an exponential phase culture and harvested at 4krpm in a benchtop centrifuge.

2) The cell pellet was washed in TNE, spun down and either snap frozen at -70°C or resuspended in 300µl TNE.

3) 10mg acid washed glass beads and 300µl phenol chloroform were added and vortexed for 2min.

4) After spinning to separate the phases, the aqueous phase was removed and extracted with phenol/chloroform (1:1) until the interface was clear.

5) The RNA was precipitated with ethanol and resuspended in 50µl dH_2O.

The yield was typically 1-2mg/ml.

2.4.5.2 Large scale.

1) A 200ml culture was grown to OD_{600} 0.3 to 0.7 before mixing with equal volumes of ice and harvesting at 4krpm at 4°C .

2) The cell pellet was washed in 9ml ice-cold TNE, resuspended in 1ml TNE and transferred to a 15ml Corex tube.

3) 30mg acid washed glass beads were added and the tube vortexed for 1min.

4) 3ml TNE, 200µl 20% (w/v) SDS and 4ml phenol were added RAPIDLY before vortexing again for 1-5min.

5) The aqueous phase was repeatedly extracted with phenol until the interface was clear.

6) The aqueous phase was extracted once with chloroform and the RNA precipitated by adding 0.1 volumes of 3M sodium acetate pH5.3 and 2 volumes ethanol.

7) After spinning at 10krpm at 4°C for 30min, the pellet was washed with 70% ethanol, air dried and dissolved in 200µl ddH_2O.

This yields 0.1-1.0mg RNA/200ml cells at OD_{600} 0.4.

RNA was stored at -20°C.

Buffers.

TNE: 50mMTris-HCl pH7.5, 5mM EDTA, 100mM NaCl.

2.4.6 Denaturing agarose gel electrophoresis of RNA.

1) 2.1g agarose was added to 15ml 10X MOPS buffer and 130.5ml dH_2O, and boiled to dissolve the agarose.

2) After cooling to below 65°C, 7.65ml formaldehyde (37%) and 7.5µl 5mg/ml ethidium bromide were added and the gel poured.

3) 30µg total yeast RNA was added to an equal volume of Formamide Sample Buffer (FSB) and heated to 65°C for 5min.

4) Samples were dry-loaded and run into the gel before covering the gel with running buffer. Empty wells at each side were loaded with agarose gel loading buffer to follow the course of the electrophoresis. The buffer was circulated by a peristaltic pump during electrophoresis.

5) Following electrophoresis, the RNA was visualised using a transilluminator and the gel photographed.

Buffers.

10X MOPS buffer: 41.86g MOPS, 6.81g sodium acetate, 3.72g EDTA. pH was adjusted to 7.0 with 5M NaOH.

FSB: 100µl 10X MOPS buffer, 200µl formamide, 120µl formaldehyde (37%).

Agarose gel loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 20% glycerol, 10mM EDTA.

2.4.7 Capillary blotting of DNA and RNA.

DNA or RNA from agarose gels was blotted onto Hybond-N membrane (Amersham) by drawing 1X SSC through the gel as described by Sambrook *et al* (1989). Prior to assembling the blot, the gels were treated as follows:

1) RNA gels were washed twice in 1X SSC for 20 min each wash.

2) DNA gels were washed twice in denaturation buffer (0.5M NaOH, 1.5M NaCl) for 20 min each wash, then washed twice in neutralising solution (0.5M Tris HCl pH7.4, 1.5M NaCl) again for 20 min each wash.

After transfer, the nucleic acids were crosslinked to the membrane by exposure to UV light in a UV Stratalinker 1800 (Stratagene) on the auto-crosslink setting.

2.4.8. Labelling restriction fragments by random priming.

Modified from Feinberg and Vogelstein (1984).

1) 2-10µg plasmid DNA was digested to completion and electrophoresed through an agarose gel.

2) The DNA fragment required was purified from the gel using Qiaex resin following the suppliers instructions.

5) 50-100ng purified fragment was made up to 35µl with ddH_2O and denatured by heating to 95°C for 5min before cooling on ice. 10µl OLB, 2µl BSA at 10mg/ml,

10μCi [α^{32}-P]dCTP and 2 units Klenow were added and the reaction incubated at room temperature overnight.

Buffers.

Solution O: 1.25M Tris-HCl pH8.0, 0.125M $MgCl_2$.

Solution A: 1ml solution O, 18μl β-mercaptoethanol, 5μl 0.1mM each dATP, dGTP and dTTP.

Solution C: Random hexadeoxyribonucleotides (Pharmacia) at 90 OD units/ml in TE.

OLB was made by mixing solutions A:B:C in the ration 2:5:3, and was stored at

-20°C.

2.4.9 Hybridisation of Southern and Northern blots with random primed probes.

1) Blots were prehybridised at 65°C in bottles in a Hybaid hybridisation oven for 2h in 10ml hybridisation buffer (Church and Gilbert, 1984).

2) The probe was added following denaturation by heating to 95°C for 5min.

3) Hybridisation was carried out at 65°C for 16-24h.

4) The membrane was washed X3 at 65°C with Church and Gilbert wash buffer.

5) The membrane was wrapped in Saran Wrap and subjected to autoradiography at

-70°C.

Buffers.

Hybridisation buffer: 7% SDS, 0.5M $NaPO_4$ pH7.2, 1mM EDTA.

Wash buffer: 5% SDS, 40mM $NaPO_4$ pH7.2, 1mM EDTA.

2.4.10 Hybridisation of blots with radiolabelled oligos.

2.4.10.1 Labelling an oligo probe.

20pmol oligo was incubated with 20μCi γ-[^{32}P] ATP, 100mM Tris-HCl pH8.0, 5mM DTT, 10mM $MgCl_2$ and 5 units T4 polynucleotide kinase for 45 min at 37°C. The enzyme was inactivated at 65°C for 10min.

2.4.10.2. Hybridising the blot.

Blots were pre-hybridised, hybridised, and washed as above. If there were any mismatches between the probe and the substrate, the hybridisation temperature was lowered by 5°C per mismatch.

2.4.11 *In Vitro* transcription.

Davanloo *et al* (1984).

1) 0.2μg linearised plasmid DNA, 1μl 10X reaction buffer, 1μl nucleotide mix, 30μCi [α^{32}P]UTP (800Ci/mmol) and 10 units RNasin (Promega) were combined in a final volume of 9.5μl.

2) 0.5μl T7 RNA polymerase was added and the reaction incubated at 37°C for 12min.

3) The reaction was stopped on ice and used directly in splicing reactions or frozen at

-20°C for up to three days.

Buffers.

10X reaction buffer: 400mM Tris-HCl pH8.0, 100mM $MgCl_2$, 100mM DTT, 100mM NaCl.

Nucleotide mix: 5mM each ATP, CTP, GTP, 0.25mM UTP (Pharmacia T7 grade).

2.4.12 Denaturing polyacrylamide gel electrophoresis of DNA and RNA.

DNA and RNA were electrophoresed through 6% (w/v) acrylamide 7M urea gels at 33W. Gels for running DNA samples (sequencing) contained 1X TB buffer, and gels for running RNA samples (splicing) contained 1X TBE buffer (section 2.1.9).

2.5 PROTEIN AND IMMUNOLOGICAL METHODS.

2.5.1 General.

2.5.1.1 Quantitation of proteins.

Protein concentration was determined using the Bradford Assay (Bradford, 1976). The dye concentrate (Bio-Rad) was used according to the suppliers instructions. BSA was used to generate standard curves.

2.5.1.2 Induction of protein by galactose.

Cultures of the strain to be induced were grown in YMGlyLacCas plus appropriate amino acids to OD_{600} 0.5, then induced by the addition of sterile 40% galactose to 2% final volume for a further 5h.

2.5.2 Extraction and SDS-polyacrylamide gel electrophoresis of yeast proteins.

2.5.2.1 Extraction of yeast total protein.

1) 100ml cultures were grown to OD_{600} 0.7 before harvesting in a benchtop centrifuge.

2) The cell pellet was washed once in TBS before being resuspended in 0.4ml TBS/NP40 and transferred to a microcentrifuge tube.

3) The cell suspension was sonicated on ice 3 times for 30sec each at 30sec intervals.

4) Cell debris was removed by spinning for 30sec.

5) The supernatant was transferred to a fresh tube and spun for 15min at 4°C to remove further debris.

6) The supernatant was transferred to a fresh tube and frozen rapidly in solid CO_2 before being stored at -70°C.

Buffers.

TBS: 50mM Tris-HCl pH8.0, 150mM NaCl.

TBS/NP40: TBS with NP40 added to 1% (v/v).

2.5.2.2 SDS PAGE of proteins.

Proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). 7.5% resolving gels were cast between 16x16cm glass plates with 1.5mm spacers, leaving room for a stacking gel and left to polymerise for 1h. The 6% stacking gel was then cast on top and the comb inserted. Protein samples were prepared by adding and equal volume of 2X sample buffer, heating to 95°C for 15min and centrifuged before loading. Gels were run at 20-30mA in SDS-PAGE running buffer.

Buffers.

Resolving gel buffer: 1.5mM Tris-HCl pH8.8, 0.4% (w/v) SDS.

Stacking gel buffer: 500mM Tris-HCl pH6.8, 0.4% (w/v) SDS.

2.5.3 Silver staining of protein gels.

Based on the method of Ansorge (1985).

Protein gels were incubated in a series of buffers at room temperature as follows.

1) 50% methanol, 12% trichloroacetic acid, 2% $CuCl_2$ for 20min.

2) Solution A for 10 min (all incubations from this point are for 10min).

3) 0.01% $KMnO_4$.

4) Solution A .

5) 10% ethanol.

6) dH_2O.

7) 0.1% $AgNO_3$ (made up immediately prior to use).

8) The gel was then washed three times with dH_2O for 20sec each.

9) The gel was washed for 1min with 10% K_2CO_3.

10) The stain was developed with up to 3 batches of developer until bands were visualised.

11) The gel was then rinsed in solution A for 1min before drying.

Buffers.

Solution A: 10% ethanol, 5% acetic acid.

Developer: 2% K_2CO_3, 0.01% formaldehyde.

2.5.4 Western Transfer.

1) After SDS-PAGE, the protein was electrophoretically transferred to Immobilon P in a Bio-Rad Trans-blot cell with two cooling coils.

2) Following transfer the membrane was stained with Ponceau S (Sigma) and rinsed with water to remove excess stain.

3) After the position of the Ponceau-stained markers were marked on, the membrane was incubated in blocking solution for 1h.

4) The blocking solution was discarded and the membrane incubated with the primary antibody (either 300µl affinity purified or 25µl crude) in 15ml blocking solution for 1h at room temperature or overnight at 4°C.

5) The membrane was washed X4 in TBS, 5min each wash, at room temperature.

6) The membrane was then incubated with the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit, Promega) at the recommended dilution (1:7500) in TBS.

7) as 5).

8) 5ml development solution containing 0.33mg/ml NBT and 0.165mg/ml BCIP was used to detect bands on the washed membrane.

9) After development, the blot was washed in water, dried and stored in the dark to minimise fading.

Buffers.

Transfer buffer: 20mM Tris base, 150mM glycine.

Blocking solution: 5% (w/v) BSA in TBS.

Development solution: 100mM Tris-HCl pH 9.5, 100mM NaCl, 50 mM $MgCl_2$.

2.5.5 Affinity purification of antibodies.

1) A strip of membrane carrying the antigen of interest was incubated with blocking solution for 2-4h at room temperature.

2) The membrane was incubated with 10ml blocking solution plus 1:100 - 1:300 dilution of polyclonal antiserum for 2-3h at room temperature of 4°C overnight.

3) The membrane was washed five times with 1X TBS/0.1% NP40.

4) The antibodies were eluted by incubating in 2ml elution solution for 5min.

5) The eluate was removed and immediately added to 0.5ml 1M Tris pH 7.5.

6) Steps 4) and 5) were repeated.

7) The eluates were pooled and dialysed against 3 changes of 1l 1X TBS for 16-20h at 4 °C.

8) The affinity-purified antibodies were stored at 4°C after the addition of 0.1% sodium azide.

2.5.6 *In vitro* splicing.

2.5.6.1 Preparation of yeast splicing extracts.

Modified from Lin *et al*, (1985).

1) 4X1l cultures were inoculated with a fresh stationary culture and grown overnight to OD_{600} 0.6-0.8.

2) The cells were spun down at room temperature and washed in 50mM potassium phosphate pH 7.5.

3) The cells were resuspended in lyticase buffer and transferred to a conical flask. 5000-8000 units of lyticase (Sigma) dissolved in lyticase buffer was added and the cells incubated with GENTLE agitation for 45min-1h.

4) Cells were checked for spheroplast formation. (A few microlitres of spheroplasted cells were incubated in either water or 1.2M sorbitol for a few minutes. On examination under a microscope, whole cells should be seen in sorbitol, and lysed cells seen in water.)

5) The spheroplasts were spun down at 3.5krpm and washed twice in 1.2M sorbitol, resuspending gently to keep the spheroplasts intact.

6) The spheroplasts were washed once in ice cold SB-3 and placed on ice. Every step from now on was carried out at 4°C.

7) The cell pellet was weighed and resuspended in 1ml buffer A for each g of cells. This suspension was transferred to a chilled Dounce homogeniser and homogenised with 12-14 strokes of the pestle.

8) The homogenate was transferred to a beaker on a magnetic stirrer at 4°C. The volume was measured and $1/9^{th}$ volume 2M KCl dropwise while stirring. The extract was stirred for a further 30min.

9) The extract was transferred to a chilled polycarbonate centrifuge tube and the cell debris removed by pelleting at 17krpm for 30min.

10) The supernatant was transferred to a chilled 50Ti polycarbonate bottle avoiding as much of the top lipid layer as possible, and also avoiding the pellet. (An optional second clearing spin can be performed at this stage.)

11) The extract was then spun at 37krpm for 1h at 4°C in a pre-cooled 50Ti rotor.

12) Again avoiding any pellet or lipid, the supernatant was transferred to dialysis tubing and dialysed for 3h against buffer D with two or three changes of buffer.

13) The extract was aliquoted into microcentrifuge tubes and frozen rapidly in solid CO_2 before storage at -70°C.

Buffers.

Lyticase buffer: 1.2M sorbitol, 50mM potassium phosphate pH7.5, 30mM DTT*.

SB-3: 50mM Tris-HCl pH7.5, 10mM $MgCl_2$, 1.2M sorbitol, 3mM DTT*.

Buffer A: 10mM HEPES-KOH pH 7.5, 1.5mM Mg Cl_2, 10mM KCl, 0.5mM DTT*.

Buffer D: 20mM HEPES-KOH pH 7.5, 0.2mM EDTA pH8.0, 50mM KCl, 20% (v/v) glycerol, 0.5mM DTT*.

*DTT was added immediately prior to use.

2.5.6.2 *In vitro* splicing.

1) Reaction mix for 10 reactions was assembled: 10µl 30% (w/v) PEG 8000 (Sigma), 8µl IgG buffer, 6µl 1M potassium phosphate pH 7.5, 2.5µl 100mM $MgCl_2$, 10µl 20mM ATP and 1000cps substrate RNA (prepared by in vitro transcription, section 2.4.13) in a final volume of 50µl.

2) The reaction mix was aliquoted into 5µl volumes and 5µl splicing extract added to each aliquot. This was incubated at 25°C for 30min before stopping the reaction on ice.

3) 2µl proteinase K solution was added to digest the protein and incubated at 37°C for 30min.

4) 100µl splicing cocktail was added and the sample extracted twice with phenol/chloroform and precipitated with ethanol after the addition of 2µg tRNA as a carrier.

5) The pellet was dried under vacuum and resuspended in 3µl Sanger dye before heating to 75°C for 2min.

6) The heated samples were briefly spun down then electrophoresed through a 6% (w/v) denaturing acrylamide gel and detected by autoradiography.

Buffers.

IgG buffer: 0.5M KPO_4 pH7.5, 33mM citrate pH 3.0, 16.67% glycerol.

Proteinase K solution: 50mM EDTA, 1% (w/v) SDS, 1mg/ml proteinase K.

Splicing cocktail: 50mM sodium acetate pH5.3, 1mM EDTA, 0.1% (w/v) SDS,

25µg/ml *E.coli* tRNA. (*E.coli* tRNA was dissolved in dH_2O and extracted with phenol/chloroform several times before being precipitated with ethanol and re-dissolved in dH_2O.)

Sanger dye: 50% (v/v) formamide, 20mM EDTA, 0.3% xylene cyanol FF, 0.3% bromophenol blue.

2.5.7 Native gel electrophoresis of splicing complexes.

Adapted from Pikielny *et al* (1986).

2.5.7.1 Pouring the gel.

150x150x1.5mm composite agarose/acrylamide gels were run in ATTO electrophoresis tanks.

1) 3-4cm blocking acrylamide (1ml 30% (w/v) acrylamide, 100µl 0.5M EDTA, 250µl 10X TB, 3.65ml dH_2O, 50µl 10% APS, 20µl TEMED) was poured into the gel plates and placed at 37°C to set. (This prevents the composite gel from sliding out of the plates during electrophoresis.)

2) 2.5ml 10XTB, 1ml 0.5M EDTA and 5ml 30% acrylamide: bisacrylamide 30:0.8was combined and placed at 65°C.

3) 0.25g agarose was dissolved by boiling in 40ml dH_2O and cooled to around 37°C.

4) At 37°C, the agarose and acrylamide solutions were combined and made up to 50ml with dH_2O at 37°C. 0.4ml 10% APS and 50µl TEMED were added, the solution mixed by inversion and poured into the plates. A comb was quickly inserted and the gel left at 37°C for around 30min.

5) The gel was chilled to 4°C overnight before use.

2.5.7.2 Running the gel.

1) The gel was pre-electrophoresed at 4°C for 30min in RNP running buffer at 80V.

2) Splicing reactions were set up as in section 2.5.6.2 as far as step 2).

3) 5µl aliquots were removed from the splicing reaction and added to 5µl Q buffer on ice.

4) 2.5µl 5X RNP loading buffer was added to each sample, loaded on the gel and run at 4°C for 8-10h at 80V or overnight at 30V.

Buffers.

RNP running buffer: 0.5X TB, 10mM EDTA.

Q buffer: 1.6M KCl, 8mM magnesium acetate, 80mM EDTA, 64mM Tris-HCl pH7.5, 2.6mg/ml yeast total RNA (prepared as in section 2.4.6 then extracted with phenol/chloroform a further 5-6 times and re-precipitated with ethanol).

5X RNP loading buffer: 5X TB, 50mM EDTA, 50% (v/v) glycerol, 0.25% (w/v) each bromophenol blue and xylene cyanol FF.

2.5.8 Preparation of ΔPRP2 ΔATP spliceosomes.

2.5.8.1 Heat inactivation of DJy85 (*prp2-1*) splicing extract.

1) 50µl DJy85 splicing extract was added to 10µl 30% PEG, 8µl IgG buffer, 6µl 1M potassium phosphate pH7.5 and 2.5µl 100mM $MgCl_2$.

2) This was incubated at 37°C for 1h.

3) For splicing, substrate RNA and ATP was added and the reaction carried out as described in section 2.5.6.2. For ΔPRP2 ΔATP spliceosomes, the following procedure was carried out.

2.5.8.2 Spliceosome assembly.

1) 100µl reactions were set up containing heat inactivated DJy85 as above, 1.5mM ATP and substrate RNA to 500cps. The reaction mix was incubated at 25°C for 30min. (5 100µl reactions provided enough spliceosomes for 10-12 splicing reactions.)

2) 1.5mM glucose (final volume) was added to the reaction mix and incubated at 25°C for a further 5min. This depleted the ATP in the extract by making use of the endogenous hexakinase activity.

3) The spliceosomes were isolated on a Sephadex G25 column (NAP-5, Pharmacia). The column was equilibrated with splicing reaction mix (section 2.5.6.2) without ATP,

and the reaction mix made up to 1ml with splicing reaction mix prior to loading on to the column.

4) The column was washed with 1-2ml of splicing reaction mix and 120µl fractions collected, numbered 1-10. The 4 or 5 hottest samples (usually fractions 3-7) containing the spliceosomes were pooled, aliquoted and either used immediately or stored at -70°C for up to 24h.

2.5.9 Immunoprecipitation.

Lossky *et al* (1987)

1) PAS beads (Sigma) were swollen in 1ml NTN for 20min and washed 5 times with NTN. 7.5mg dry mass (50µl swollen beads) were used per reaction.

2) The antibody was added to the beads at sufficient titre to give maximal precipitation of antigen, and incubated in 300µl NTN with mixing for 1h. In this case, 90µl anti-54 was used per reaction.

3) 200µl freshly made blocking buffer was added and incubated with mixing for a further 45 min.

4) The beads were washed 5 times with NTN and placed on ice until the splicing reaction was ready.

5) A 10µl splicing reaction was set up and taken through to step 2 (section 2.5.6.2).

10µl ice cold Q buffer was added and NTN was added to give a final volume of 300µl. This was added to the antibody-bound PAS beads and incubated at 4°C for 2h with mixing.

6) The beads were washed several timed with washes of 1ml NTN and monitored until the signal from the negative control is minimal. The beads were then washed once in NT.

7) To analyse RNA, 50µl proteinase K solution was added and incubated at 37°C for 30min with mixing.

8) 50µl dH2O was added and the sample extracted 3 times with phenol/chloroform.

9) The RNA was precipitated with ethanol adding 1µl E.coli tRNA at 20mg/ml as a carrier.

10) The samples were electrophoresed through a 6% (w/v) denaturing acrylamide gel before exposure to autoradiography at -70°C.

Buffers.

Blocking buffer: 100µg/ml each of BSA, glycogen and *E.coli* tRNA, used fresh.

Q buffer: (check recipe for RNP gels)

NT: 150mM NaCl, 50mM Tris-HCl pH7.5.

NTN: as NT but with 0.1% (v/v) NP40.

Proteinase K solution: 50mM Tris-Hcl pH7.5, 300mM NaCl, 5mM EDTA, 1.5% (w/v) SDS, 2mg/ml proteinase K (stored at -20°C).

2.5.10 Purification of polyhistidine-tagged Prp2 protein from yeast.

2.5.10.1 Preparation of splicing extracts for purification of Prp2 protein.

1) S150 cells carrying the inducible allele of *PRP2* to be purified on a plasmid were grown in YMGlyLacCas and induced as described in section 2.5.1.2.

2) The splicing extract described in section 2.5.6.2 was carried out except that no DTT was added to buffer a or SB-3, and the final dialysis step into buffer D was omitted. The extract was snap frozen and stored at -70°C before purification.

2.5.10.2 General guidelines.

Columns were run at 4°C. All buffers were cooled to 4°C before use. A peristaltic pump was used to control the flow rate through the columns. The volume referred to in the following sections is the volume of packed resin in the column. A 1.77cm^2 (5cm long) column was used for the nickel-chelating column, and a 0.2cm^2 (5cm long) column was used for the polyuridylic acid agarose column.

2.5.10.3 Nickel chelating column.

Hoffman and Roeder (1991).

1) Packed Ni-NTA resin was washed with 40ml pre-wash at a flow rate of 20ml/h for 2h.

2) Splicing extract was added to an equal volume of 2X sample buffer and 1mM imidazole and circulated through the column for 3-4h at a flow rate of 15ml/h.

3) The column was washed with 30ml 20mM imidazole pH7.9 in BC100 at 15ml/h.

4) The column was washed with 20ml 50mM imidazole as in step 3.

5) Protein was eluted from the column in 5ml 150mM imidazole followed by 5ml 200mM imidazole at a flow rate of 5ml/h.

Buffers.

Pre-wash: 20mM Tris-HCl pH7.9, 0.5M NaCl.

2X sample buffer: 20mM Tris-HCl pH7.9, 1M NaCl, 20% glycerol.

BC100: 200mM Tris-HCl pH7.9, 100mM KCl, 20% glycerol.

1M imidazole was made up in dH_2O and pH adjusted to pH7.9 with HCl before dilution with BC100 to make 20, 50, 150 and 200mM solutions.

2.5.10.4 Polyuridylic acid agarose column.

Adapted from Kim *et al* (1992).

1) 300mg polyuridylic acid (poly(U)) agarose was swollen in 3ml BP buffer for 30min with mixing.

2) Swollen poly(U) was packed in to form a 1ml column.

3) The column was washed with 20ml BP containing 50mM KCl at a flow rate of 15ml/h.

4) Peak fractions (as identified by Western blotting) from the Ni-NTA column, which had been pooled and dialysed against buffer D (section 2.5.6.1) for 2h at 4°C with a change of buffer after 1h, were circulated over the column at a flow rate of 15ml/h for 2h.

5) The column was washed with 20ml BP plus 100mM KCl followed by 10ml BP containing 150mM KCl at a flow rate of 15ml/h.

6) Prp2 protein was eluted with 5ml BP/200mM KCl and 5ml BP/250mM KCl at a flow rate of 5ml/h. 0.5ml fractions were collected and analysed by western blotting to identify peak samples and silver staining to assess purity.

Buffers.

BP: 20mM HEPES-KOH pH7.7, 0.5mM EDTA, 0.01% NP40, 10% glycerol, 5mM DTT*.

* DTT was added immediately before use.

2.5.11 β-galactosidase assays.

Miller, 1972.

1) Cells were induced if necessary (section 2.5.1.2) and 1ml aliquots harvested in mid log phase.

2) Cells were resuspended in 0.5ml Z buffer.

3) 25µl $CHCl_3$ was added and vortexed for 20sec.

4) 100µl ONPG was added and incubated at 23°C for 30min-5h.

5) The reaction was stopped by the addition of 250µl 1M Na_2CO_3.

6) The cell debris was spun down and the supernatant assayed at 420nm.

Buffers.

Z buffer: 60mM Na_2HPO_4. 40mM NaH2PO4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol, 0.025% (w/v) SDS.

ONPG: 4mg/ml ONPG in 0.1M potassium phosphate buffer.

β-galactosidase activity was calculated by the following formula.

$$\text{units of } \beta\text{-galactosidase activity} = \frac{OD_{420}}{(t \times OD_{600} \times V)} \times 1000$$

Chapter three.

Analysis of the zinc finger motif of *PRP2*.

3.1 Introduction.

Zinc finger motifs are known to be important in nucleic acid/protein binding. As splicing is a highly dynamic process involving a changing pattern of RNA/protein interactions, it is conceivable that RNA binding motifs including zinc fingers will be involved throughout the splicing process, and indeed, it has been shown that Prp9 protein has zinc finger sequences required for splicing (Legrain and Choulika, 1990) and functions as part of a complex implicated in the addition of the U2 snRNA to the spliceosome (Legrain and Chapon, 1993). A similar motif is found in Prp11 protein (Chang *et al*, 1987).

The function of the zinc finger domains of Prp9 and Prp6 proteins were investigated by Legrain and Choulika using site-directed mutagenesis and complementation of temperature-sensitive mutant strains. With Prp9, they found that by mutagenising the first histidine of the first zinc finger domain to leucine, or any cysteine or histidine in the second zinc finger domain to serine or leucine respectively, ability to complement the ts defect of the *prp9* strain was lost. The cysteine and histidine residues of Prp6 protein were also mutagenised to serines and leucines respectively. In this case, plasmids carrying *PRP6* with any one of the four positions mutated were able to complement a *prp6* ts strain at the non-permissive temperature.

The protein sequence of Prp2 contains a zinc finger-like motif towards the C-terminus. This motif is unusual in that instead of pairs of cysteine residues and histidine residues, it has three cysteine residues and one histidine. The alignment of the zinc finger-like sequence of Prp2 with other such sequences is shown in table 3.1.

Table 3.1 Alignment of the zinc finger region of Prp2 protein with other similar motifs.

Zn finger consensus (TFIIIA)	Y/F-	C --- C (2-4)	---F-----L--	H ---- H (3-4)	
Prp2	**GTITRLGKMM**	**C EFP C**	**EPEFAKVLYTAAT**	**H EQ C**	**QGVLEECLTI**
Prp6	SVAKKIIENG	C QE C	PRSSDIWLENIRL	H ESDV H	YCKTLVATAI
Prp9	SLRSEAKGIY	C PF C	SRWFKT S SVFES	H LVGKI H	KKNESKRRNF
	KLHGLDREYR	C EI C	SNKVYNGRRT FER	H FNEER H	IYHLRCLGIE
Prp11	IYKNHSGKLV	C KL C	NTMHMSWS SV ER	H LGGKK H	GLNVLRRGIS

Table 3.1 Sequences from Prp2 protein (Chen and Lin, 1990), Prp6, Prp9 (Legrain and Choulika, 1990) and Prp11 proteins (Chang *et al*, 1988) are aligned with the consensus sequence of zinc finger motifs as derived from TFIIIA (Vincent, 1987).

3.2 Results

3.2.1 Mutagenesis of the second cysteine residue of the zinc finger motif results in a non-functional Prp2 protein.

To investigate the zinc finger motif in the Prp2 protein, *in vitro* mutagenesis was used to make changes in *PRP2* resulting in substitutions of the cysteine and histidine residues in the zinc finger motif.

The first mutation made to investigate the function of the zinc finger motif was a change of the second cysteine residue to tyrosine (Figure 3.1). The mutation, made by Erica Whittaker, was introduced by site-directed mutagenesis in M13 as described in section 2.4.4, using M13T3B as a template and oligo 026A. The resultant mutant construct was called M13P2C2.

Figure 3.1 Wild-type sequence of the zinc finger motif of Prp2 protein showing position of the P2C2 mutation.

```
GTITRLGKMM     C EFP C     EPEFAKVLYTAAT      H EQ C     QGVLEECLTI
                       ↓
                       Y
```

Figure 3.1. The second cysteine residue of the putative zinc finger was changed to tyrosine by site-directed mutagenesis.

3.2.2 Construction of a galactose-controllable mutant allele.

In order to investigate the behaviour of the P2C2 mutant, a galactose-inducible construct was used. pBMPRP2 contains the *GAL1* promoter. This allows control over the expression of *PRP2* by growing on glucose, which represses expression, or growing on galactose, where expression is induced, allowing the allele to be overexpressed.

Figure 3.2. Sequence of pTZP2C2 showing the G to A mutation.

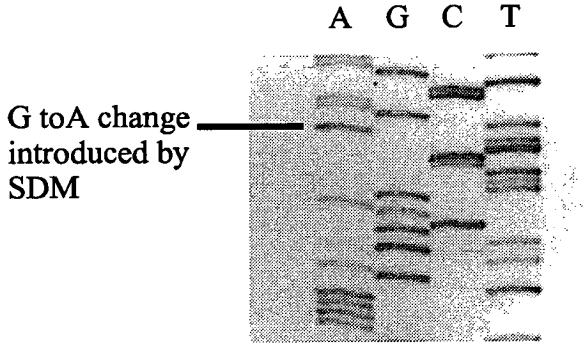


Figure 3.2 A 2.4kb *Hin*dIII-*Xba*I fragment was subcloned from pBMP2C2 recombinants into pTZ19R. Template DNA from pTZP2C2 was sequenced to confirm the presence of the G to A mutation.

The *Pvu*II-*Xba*I fragment of pBMPRP2 was removed and replaced with the *Pvu*II-*Xba*I fragment of M13P2C2. This procedure carries some risk that resultant colonies contain the parent pBMPRP2 plasmid and not the recombinant pBMP2C2. These are indistinguishable by restriction digest. To confirm the identity of recombinant clones, a 2.4kb *Hin*dIII-*Bam*HI fragment from possible pBMP2C2 clones containing the zinc finger motif was subcloned into pTZ19R. Single stranded DNA was prepared from the

Figure 3.1 Wild-type sequence of the zinc finger motif of Prp2 protein showing position of the P2C2 mutation.

```
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                       ↓
                       Y
```

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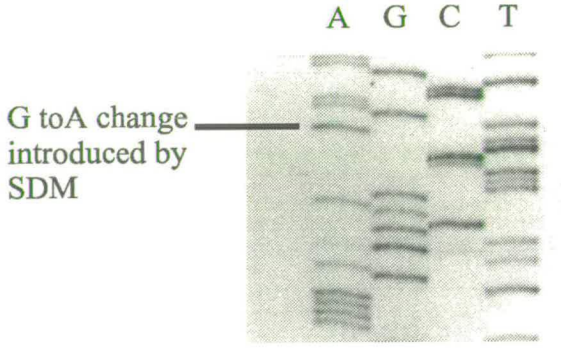

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recombinant pTZ constructs and sequenced using oligo 027A as a primer. Figure 3.2 shows the mutant sequence containing the G to A change.

3.2.3 Complementation of a *prp2-1* temperature-sensitive strain.

pBMPRP2, pBMP2C2 and pBM125 were transformed into the yeast strain DJy36. DJy36 is a *prp2-1* strain which is temperature-sensitive, only growing at or below 30°C. The transformant colonies were purified and spotted onto selective glucose and galactose plates at 23°C and 36°C.

Figure 3.3 shows that although the pBMPRP2 plasmid can complement DJy36 at 36°C on glucose and galactose, pBMP2C2 cannot complement the *prp2-1* defect.

Figure 3.3. Complementation assay.

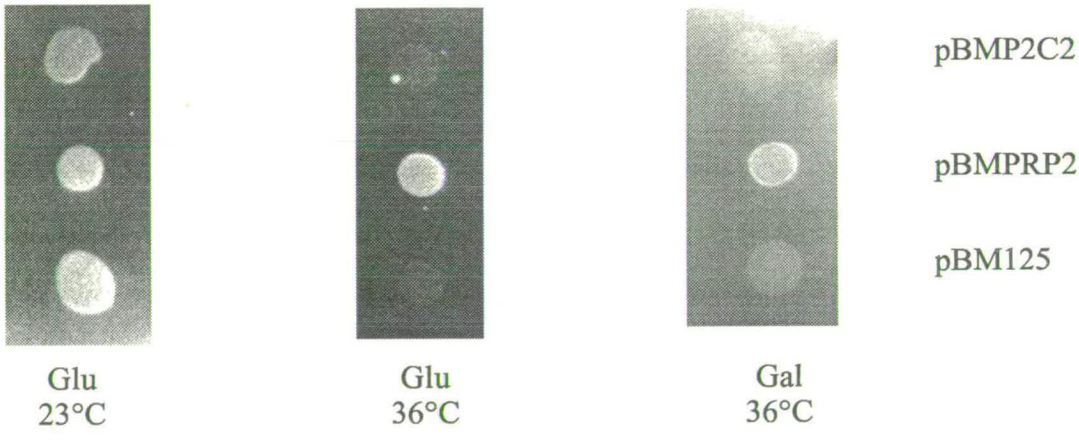


Figure 3.3. Transformants of a *prp2-1* ts strain, DJy36, carrying pBMP2C2, pBMPRP2 (positive control) or pBM125 (negative control) were spotted onto galactose plates and grown at the pemissive (23°C) or non-permissive (36°C) temperature. The cells transformed with pBMP2C2 fail to grow at the non-permissive temperature, so the P2C2 mutant fails to complement the *prp2-1* ts defect.

Transformants on plates incubated at 30°C and below grow, indicating that pBMP2C2 plasmid does not confer cold sensitivity on the cells.

To check that the absence of complementation by pBMP2C2 was not due to lack of expression of the protein, western analysis of cultures grown under inducing and non-

inducing conditions was carried out. Western analysis (figure 3.4) shows that the mutant protein is overexpressed in the presence of galactose, so lack of complementation is not due to absence of the protein.

Figure 3.4 Western blot showing induction of pBMP2C2 by galactose.

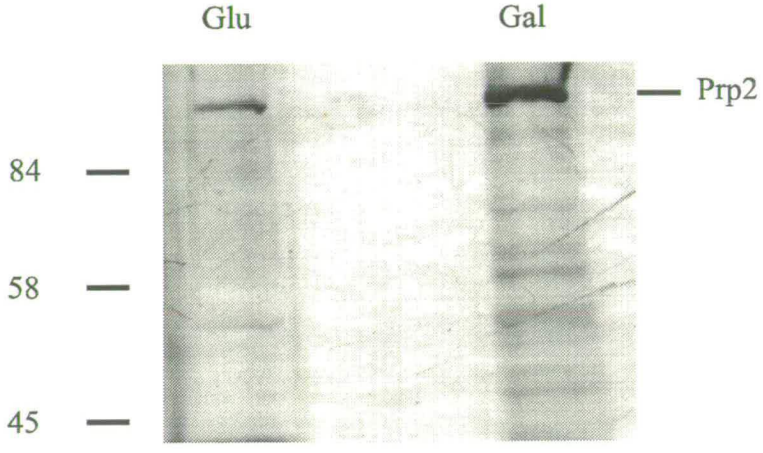


Figure 3.4. Cultures of DJy36 transformed with pBMP2C2 were grown in selective glucose (Glu) or galactose (Gal) medium. Equal amounts of extract as determined by Bradford assay were separated by SDS-PAGE and transferred to Immobilon-P by electroblotting. The membrane was probed with anti-140 antiserum.

3.2.4. Construction of pSA1, pSA2 and pSA3 as cloning vehicles.

To facilitate the construction of further recombinant mutant constructs and avoid possible confusion with the wild-type parent plasmids, a series of linker constructs were made. Using restriction sites unique to *PRP2*, fragments of *PRP2* were removed from pBMPRP2 and replaced by short linkers (see table 3.2).

Table 3.2. Regions of pBMPRP2 replaced by oligos to form linker constructs

Plasmid	Region of *PRP2* removed	Linker	Oligos forming linkers
pSA1	579bp *Pvu*II-*Sau*I fragment	*Pvu*II-*Sac*I-*Sau*I	449J/450J
pSA2	582bp *Xba*I-*Pvu*II fragment	*Xba*I-*Sac*I-*Pvu*II	446K/447K
pSA3	778bp *Hin*dIII-*Xba*I fragment	*Hin*dIII-*Sac*I-*Xba*I	484K/485K

For construction of subsequent mutant plasmids, the appropriate linker construct was cut with the two enzymes that cleave at either end of the linker. Following ligation with the mutant fragment, the recombinant DNA could be incubated with *Sac*I prior to transformation, thus reducing the background of non-recombinant constructs. Recombinants could easily be distinguished from the linker construct by restriction analysis.

3.2.5 Further mutagenesis of the zinc finger motif.

Further site-directed mutagenesis was carried out to characterise the zinc finger of the Prp2 protein. As shown in table 3.3, the C2, C3 and C4 residues were changed to serine, a more conservative change than tyrosine. The histidine residue was changed to leucine, also a conservative change. As there is a possibility that the C4 residue may compensate in the event of C3 being mutated, a double mutant was also constructed which contained both C3 and C4 residues mutated to serines.

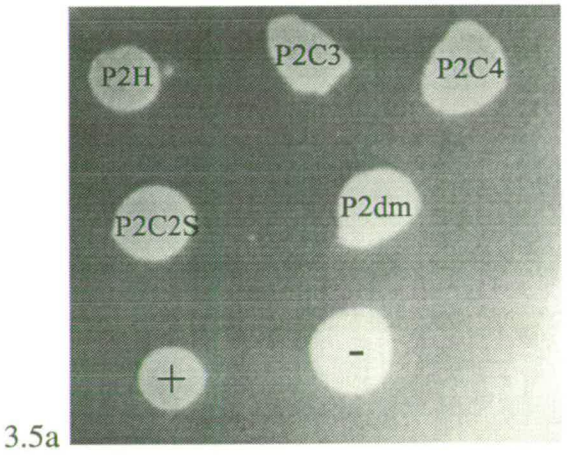
Table 3.3 The zinc finger sequence of Prp2 protein and position of mutations.

wild-type sequence	GTITRLGKMM	C EFP C	EPEFAKVLYTAAT	H EQ C	QGVLEECLTI
cysteine residue number		1 2		3	4
mutation:					
P2C2S		S			
P2C3				S	
P2C4					S
P2dm				S	S
P2H				L	

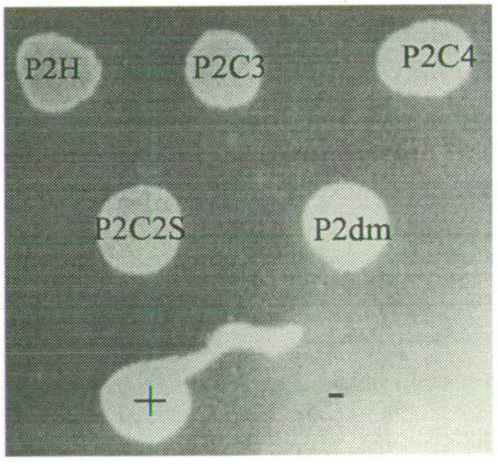
The C2S mutation was made by the Kunkel method of site-directed mutagenesis using a pTZPRP2 template.

The remaining mutations were made by PCR using oligos which primed at the *Pvu*II site carrying the mutations, and a wild-type oligo priming from the 3' *Bam*HI site. The PCR products were cloned into pTZ19R by cutting with *Pvu*II and *Bam*HI, and sequenced to confirm the presence of the mutation. As *Taq* polymerase has a relatively low fidelity rate, the whole PCR fragment was sequenced to ensure that no mutations were present other than the desired change.

Figure 3.5. Complementation of a prp2-1 ts strain by zinc finger mutants.



3.5a



3.5b

Figure 3.5. DJy36 (*prp2-1*) was transformed with pBMP2-series plasmids carrying mutations as detailed in table 3.3. Colonies from purified transformants were suspended in sterile water and spotted onto selective plates. A transformant carrying pBMPRP2 was used as the positive control (+), which grows at all temperatures, and one of pBM125 was used as the negative control (-), which grows at or below 30°C. 3.5a shows that all the tranformants grow on glucose at the permissive temperature, 23°C. Figure 3.5b shows that at the non-permissive temperature, 36°C, background expression from the *GAL1* promoter is sufficient for all the point mutants to rescue the ts defect of DJy36 on glucose. Cells transformed with the negative control fail to grow at this temperature.

In the case of the double mutant, PCR was carried out using a template containing the C4-S mutation, the C3-S mutation being introduced by oligo164H during the second round of PCR.

After sequencing confirmed the presence of the desired mutations, *Pvu*II/*Sau*I mutant fragments were ligated into the pSA1 linker construct.

The new mutant constructs were transformed into the *prp2-1* strain DJy36 and tested for complementation on glucose and galactose. Figure 3.5 shows that all the mutants can complement the *prp2-1* ts defect even on glucose medium at the non-permissive temperature, 36°C.

3.2.6 Insertion of the JB1 oligo adds four amino acids to the loop of the zinc finger.

It was decided to further investigate the role of the putative zinc finger in the Prp2 protein by inserting an oligo into the *Pvu*II site in the zinc finger loop.

The JB1 oligo is self-complementary so was annealed and ligated into the *Pvu*II-cut pBMPRP2. This inserts four additional amino acids into the loop of the zinc finger (figure 3.6). The insertion carries a diagnostic *Cla*I site which was used to screen recombinant plasmids for insertions. Cutting the recombinant pBMPRP2ins with *Cla*I confirmed that the insertion was present.

Figure 3.6 *Cla*I insert oligo and surrounding sequence of *PRP2*.

DNA sequence TAT A**CA G***CT AAT CGA TTA G***CT** GCT ACG

Protein sequence Y T *A N R L* A A T

Figure 3.6. Sequence of *PRP2* with the *Pvu*II site highlighted in bold type. The oligo insertion is indicated in italics. Amino acid sequence is shown below each codon with inserted residues in italics. The diagnostic *Cla*I site in the oligo is underlined.

To confirm the insertion of one oligo repeat, a 2.4kb *Hin*dIII/*Bam*HI fragment was subcloned into pTZ19R and sequenced using oligo 1970. This verified the presence of one copy of oligo.

3.2.7 pBMPRP2ins is unable to fully complement a temperature-sensitive *prp2-1* strain.

pBMPRP2ins was transformed into the temperature-sensitive *prp2-1* strain DJy36. When expression of the mutant gene was induced by growth on galactose plates, this construct complemented the temperature-sensitive defect of *prp2-1* up to 34°C but not above (figure 3.7). Under repressing conditions on glucose plates, no complementation was seen, even although the wild-type construct is known to complement up to 36°C on glucose. This complementation by the wild-type allele is due to incomplete repression of the *GAL1* promoter by glucose.

Figure 3.7 Complementation of DJy36 by pBMP2ins

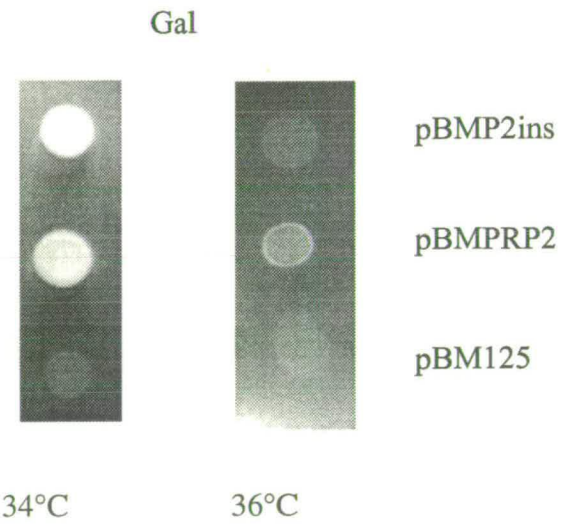


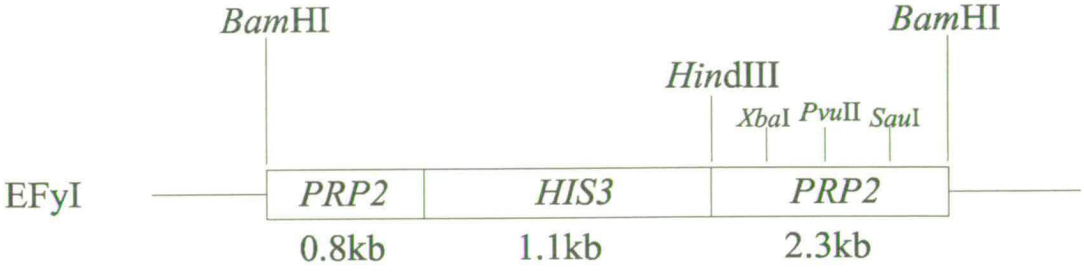
Figure 3.7. Transformants of the temperature-sensitive strain, DJy36, were suspended in sterile water and spotted onto selective galactose plates as before. Plates incubated at 34°C showed complementation by pBMP2ins, but plated incubated at 36°C did not.

3.2.8 Construction of SAy1 disrupted diploid.

As the complementation assays seemed to give contradictory results , it was decided to assay the mutants another way, by replacing the genomic copy of *PRP2* with the mutant alleles. As *PRP2* is an essential gene, gene disruption and replacement is carried out in

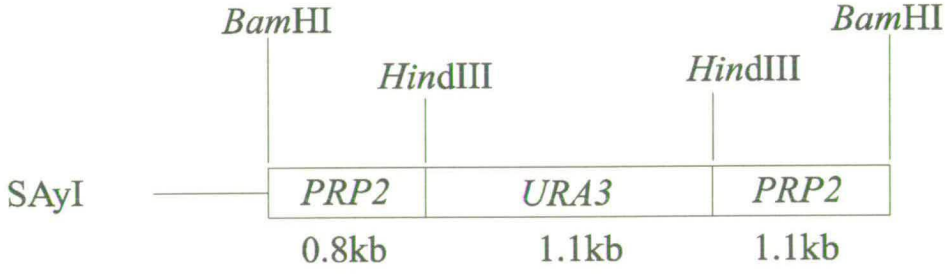
a diploid strain in which only one of the *PRP2* alleles is disrupted. The phenotype of the replaced gene can then be assayed after sporulation and tetrad analysis.

Figure 3.8 Constuction of SAy1.



EFyI has one chromosomal locus disrupted by *HIS3* inserted at the *Hin*dIII site. *PRP2* sequence contains the zinc finger motif (on either side of the *Pvu*II site).

To remove the zinc finger sequence, this diploid was transformed with the 3kb *Bam*HI fragment from the plasmid pEF2Δ, which carries *PRP2* disrupted by *URA3* and has the *Xba*I-*Sau*I fragment removed. Transformants were selected on ura- plates and subsequently screened for his- phenotype. One such isolate was named SAyI.



A previously constructed diploid carried a disruption that still contained the zinc finger region of the *PRP2* ORF. To facilitate construction of a strain to assay the zinc finger mutants, a diploid strain had to be constructed in which the disrupted locus did not contain the zinc finger sequence. A strategy was developed to delete some of the *PRP2* sequence from the plasmid pEF2, which contains *PRP2* disrupted by *URA3*.

An *Xba*I/*Sau*I fragment was cut out of pEF2, the ends of the plasmid filled in and ligated together. This plasmid was called pEF2Δ. The *Bam*HI fragment from pEF2Δ containing the disrupted *PRP2* was gel purified and transformed into the diploid strain EFy1 (**a**/α, *ura3-52/ura3-52, his3-Δ200/his3Δ1, ade2-101oc/ADE2, lys2-801/LYS2, trp1-Δ1/trp1-289, LEU2/leu2-3,112, PRP2/prp2::HIS3*) (figure 3.8). EFy1 carries a

prp2 allele disrupted by *HIS3* and one wild-type *PRP2* locus. The resulting transformants were tested for the desired phenotype: trp$^-$, his$^-$ and Ura+. A Southern blot of genomic DNA probed with a fragment of *PRP2* (figure 3.9, track 8) shows a band of the appropriate size. This strain was called SAy1.

The genotype of SAy1 is as follows:

a/α, *ura3-52/ura3-52, his3-Δ200/his3Δ1, ade2-101^{oc}/ADE2, lys2-801/LYS2, trp1-Δ 1/trp1-289, LEU2/leu2-3,112, PRP2/prp2::URA3.*

3.2.9 Behaviour of zinc finger mutants on the genome.

To place the zinc finger mutants on the genome, a 3kb *Bam*HI fragment from each pBMP2-series mutant construct was gel-purified twice using the Qiaex system. The purified mutant fragments were transformed into SAy1 and grown on the appropriate selective medium containing 5-FOA to select against Ura$^+$ transformants.

Transformants were tested for the appropriate markers by growth on minimal medium lacking either uracil, histidine or tryptophan. Desired transformants were ura$^-$, his$^-$ and trp$^-$. A control transformation with no DNA was carried out. Southern analysis was carried out for each diploid strain before sporulation (figure 3.9). This showed that the disrupted *prp2::URA3* band seen in the untransformed parent strain had been replaced by one full length *PRP2* band in each transformant.

Figure 3.9 Southern blot of genomic DNA from SAy1 following gene replacement with zinc finger mutations.

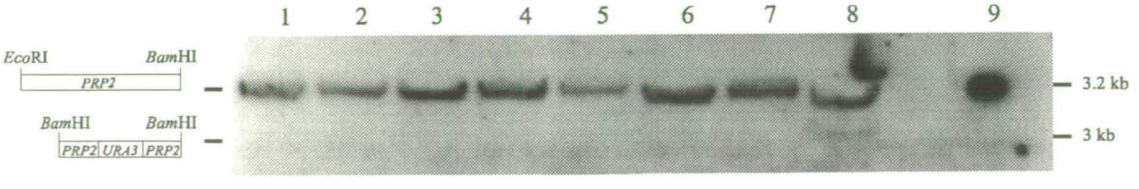


Figure 3.9. Genomic DNA was isolated from diploid strains following gene replacement and digested with *Eco*RI and *Bam*HI. Following electrophoretic separation, the DNA was

transferred to Hybond-N and the membrane probed with a 3kb *Bam*HI fragment of *PRP2*. The top band corresponds to the 3.2kb *Eco*RI-*Bam*HI fragment of *PRP2* which includes the *PRP2* promoter, the lower fragment corresponding to the disrupted *prp2::URA3*. Tracks are as follows: 1. P2C4; 2. P2H; 3. P2ins; 4. P2C2; 5. P2C2S; 6. P2dm; 7. P2C3; 8. SAy1; 9. 3kb *PRP2* fragment from pBMPRP2 plasmid.

Tetrads were prepared from each transformed diploid by growing on sporulation medium for 3-4 days. Asci were scraped off the plate and resuspended in spore buffer as in section 2.3.6. Between 5 and 10 individual tetrads for each strain were dissected using a Singer MSM micromanipulator and the dissected spores grown on YPDA for 3-4 days. Each tetrad yielded four viable spores. Auxotrophic markers were checked by growth on minimal medium to verify 2:2 segregation. All incubations up to this stage were carried out at 23°C.

All haploid progeny derived for each tetrad were spotted onto plates and incubated at 18 °C, 23°C, 30°C, 34°C and 36°C. The mutant alleles P2C2 and P2ins, which on plasmids had failed to complement the *prp2-1* defect in DJy36, supported growth up to 36°C when present as the chromosomal locus. Following this result, tetrads were dissected for another individual transformant of each mutant. Again, complementation was seen at the non-permissive temperature. Progeny resulting from the other mutants, P2C2S, P2C3, P2C4, P2dm and P2H, were tested and grew at all temperatures.

3.3 Discussion

The P2C2 and P2ins mutants showed some loss of function in the *prp2-1* complementation assay. Other substitutions of cysteine and histidine residues had no effect on the ability to complement the *prp2-1* ts defect of DJy36. It was considered that the full effect of mutations may not be observed in a *prp2-1* genetic background due to low level activity of the ts allele at the restrictive temperature, or possibly as a result of intragenic complementation. The ability of the mutant alleles to support growth as the sole copy of *prp2* was therefore tested.

The P2C2 and P2ins mutants support growth when they are the sole sources of *PRP2* in the cell (section 3.2.9). This seems contradictory to the initial result of not fully

complementing the ts defect of the *prp2-1* strain. One possible explanation for this is that in a *prp2-1* strain, the introduced mutant protein competes poorly with the prp2-1 protein for interaction with spliceosomes. As the insertion mutant is slightly larger than the prp2-1 protein, the prp2-1 protein may compete more effectively for the spliceosomal binding site. In the case of the P2C2 mutant, the non-conservative substitution may result in a conformational change which interferes with the ability to enter the spliceosome to a greater extent than the more conservative substitutions. This may result in a greater defect in the protein which is manifested by the inability to complement the *prp2-1* mutation. When these mutants are the sole source of Prp2 protein in the cell, there is no competition so the cells are able to grow at all temperatures. Immunoprecipitation experiments with anti-Prp2 antibodies failed to show association of prp2-1 protein with the spliceosome (King and Beggs, 1990). However, this may be due the mutant protein being in a conformation not recognised by the antibodies.

Another difference between the complementation of the ts defect and genomic expression of *PRP2* is that in the former case the mutant proteins are being highly produced. It is conceivable that the mutant proteins become dominant negative at temperatures above 30°C when overproduced despite being functional at normal levels of expression and at lower temperatures. One possibility might be that the transient interaction of Prp2 with the spliceosome is critical such that an interacting factor must be made available for a different interaction subsequent to Prp2 function. If the mutant protein has an altered affinity for this factor, overproduction combined with higher temperatures could be detrimental.

An interesting experiment would be to construct a series of mutants with multiple inserts of the JB1 oligo and screen for an exaggerated phenotype.

In the case of the other mutants, i.e. P2C3, P2C4, P2H, P2C2S and P2dm, it was theoretically possible that the function of these proteins was affected to some minor degree but this was not manifested as a ts defect in the presence of prp2-1 protein. However, as these proteins appear fully functional as the sole source of Prp2 in the cell, complementation by heterodimerisation or intragenic complementation is ruled out.

These "silent" mutations may prove synthetic lethal in combination with mutant co-factors, as yet unidentified, or even with intragenic mutations.

Zinc fingers rarely work in isolation, as there are usually several consensus sequences within the one protein eg. TFIIIA which has 9, so it is a possibility that another domain in Prp2 is capable of compensating for some loss of function of the zinc finger. The Prp2 protein is known to bind RNA although the exact RNA binding site is unknown (Plumpton *et al*, 1994; Teigelkamp *et al*, 1994).

When the RNA binding site of Prp2 is elucidated, it would be interesting to combine mutations in the RNA binding site with mutations in the zinc finger. In the event of single mutations in the RNA binding site having no detectable phenotype, it is possible that combined RNA-binding/zinc finger mutants would exhibit a gross detectable defect, or that a subtle phenotype may be exacerbated by combining mutations.

Many examples of redundant structures in the spliceosome are coming to light. In the case of snRNAs, highly conserved sequences most notably in the U2 and U6 snRNAs can be mutated with no effect on the cell growth. The U2/U6 helix II involves base pairing between the 5' end of the U2 snRNA and the 3' terminal domain of the U6 snRNA. This structure would appear to be important for the splicing process in mammalian cells (Datta and Weiner, 1991, Wu and Manley, 1991). In yeast, mutations in the U6 snRNA that have little or no effect on cell growth (Fabrizio *et al*, 1989, Madhani *et al*, 1990) were later found to disrupt this helix (Madhani and Guthrie, 1992, Hausner *et al*, 1990). More recently, the U2/U6 helix III has been described (Yan and Ares, 1996). Single mutations in U2 which disrupt this helix have no effect on cell growth, but double and triple mutants were found to be lethal. Other highly conserved sequence elements in yeast which appear to be dispensable include the stem-loop I of the U1 snRNA (Liao *et al*, 1990) and the base pairing between U4 and U6 (Fabrizio and Abelson, 1990).

In the case of spliceosomal proteins, mutations in different domains of Uss1 protein, a U6-specific splicing factor, inactivate the function of the protein when in combination, but not individually (M. Cooper, unpublished results).

It remains a possibility that the zinc finger motif in Prp2 is similarly redundant and the function has been taken over by another splicing factor. In this scenario, the P2C2 and P2ins insertion mutants may cause disruption to the conformation of the Prp2 protein, resulting in the observed phenotype. The other more conservative mutations may not be so disruptive to the protein's conformation and are therefore silent.

Chapter four.

Deletion analysis of dominant negative alleles of *PRP2*.

4.1 Introduction

As the Prp2 protein has a transient interaction with the spliceosome, conditional dominant negative mutations were generated which froze this interaction, leaving Prp2 stably associated with spliceosomes which are unable to undergo step one. When these dominant negative mutant proteins are introduced they compete with wild-type Prp2 for interaction with the spliceosome. If the mutant protein interacts with the spliceosome, it forms a stable interaction and splicing cannot proceed. As the expression of these mutant alleles is regulated by the *GAL1* promoter, the strain can be propagated on glucose, and dominant negative activity induced by galactose. These dominant negative alleles facilitate the study of the interaction between Prp2 and the spliceosome.

Figure 4.1. Restriction map of *PRP2* showing position of mutations in conserved sequences and areas deleted.

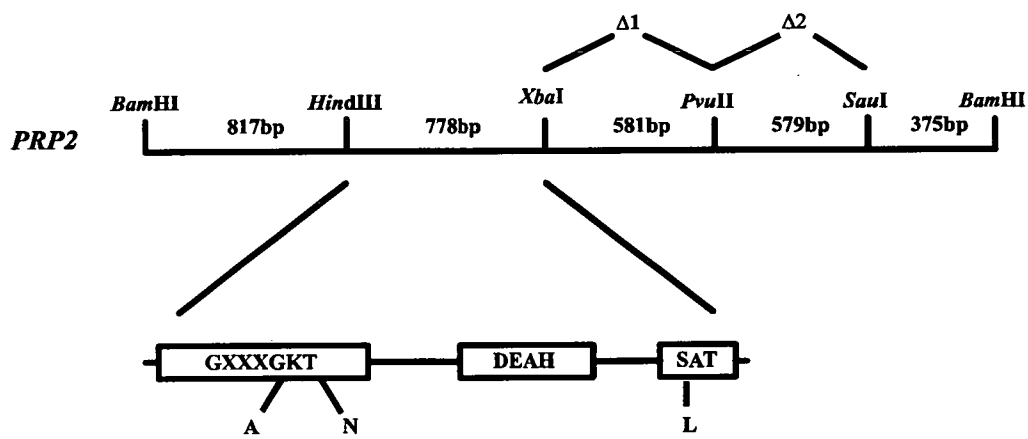


Figure 4.1. Mutations were made in conserved sequences changing GKT to AKT and GKN, and changing SAT to LAT.

The dominant negative mutants were generated in the conserved sequences shown in figure 4.1; one changing serine to leucine in the SAT box ($Prp2^{LAT}$) was made by Mary

Plumpton, and two mutations in the ATP-binding site, glycine to alanine ($Prp2^{AKT}$) and threonine to asparagine ($Prp2^{GKN}$) were made by Elizabeth Flinn.

When these proteins were overproduced in a wild-type background and a splicing extract made, they were found to inhibit splicing *in vitro.* Immunoprecipitation experiments, in which ^{32}P-labelled pre-messenger RNA is incubated with splicing extracts under splicing conditions, then precipitated with anti-Prp2 antibodies, showed after electrophoresis of the labelled RNA that the $PRP2^{LAT}$ and $PRP2^{GKN}$ mutant proteins form a stable association with the spliceosome (Plumpton *et al*, 1994; E. Flinn,1994).

In experiments designed to map the the RNA binding site of the Prp2 protein, deletions were made within dominant negative alleles.

4.2 Results

4.2.1. Deletion analysis of $Prp2^{LAT}$, $Prp2^{GKN}$, $Prp2^{AKT}$ proteins.

To investigate which part of the proteins is necessary, in addition to the mutation, for dominant negative activity, possibly an RNA binding site, deletions were made downstream of the conserved region of *PRP2*, from *Xba*I to *Pvu*II, or from *Pvu*II to *Sau*I (figure 4.1). This was carried out in pBMPRP2 carrying $Prp2^{LAT}$, $Prp2^{GKN}$, and $Prp2^{AKT}$ mutations. These constructs were transformed into S1502B, a strain wild-type for *PRP2,* and tested for dominance in the presence of galactose.

Figure 4.2 shows that when the full-length dominant negative proteins were overproduced in a wild type background at 30°C, the cells were unable to grow, but cells which expressed the shortened dominant negative alleles of $Prp2^{LAT}$, $Prp2^{GKN}$, and $Prp2^{AKT}$ all grew. That is, the deletions resulted in loss of dominance at 30°C.

Figure 4.2. Assay for dominant negative activity of shortened *PRP2* alleles.

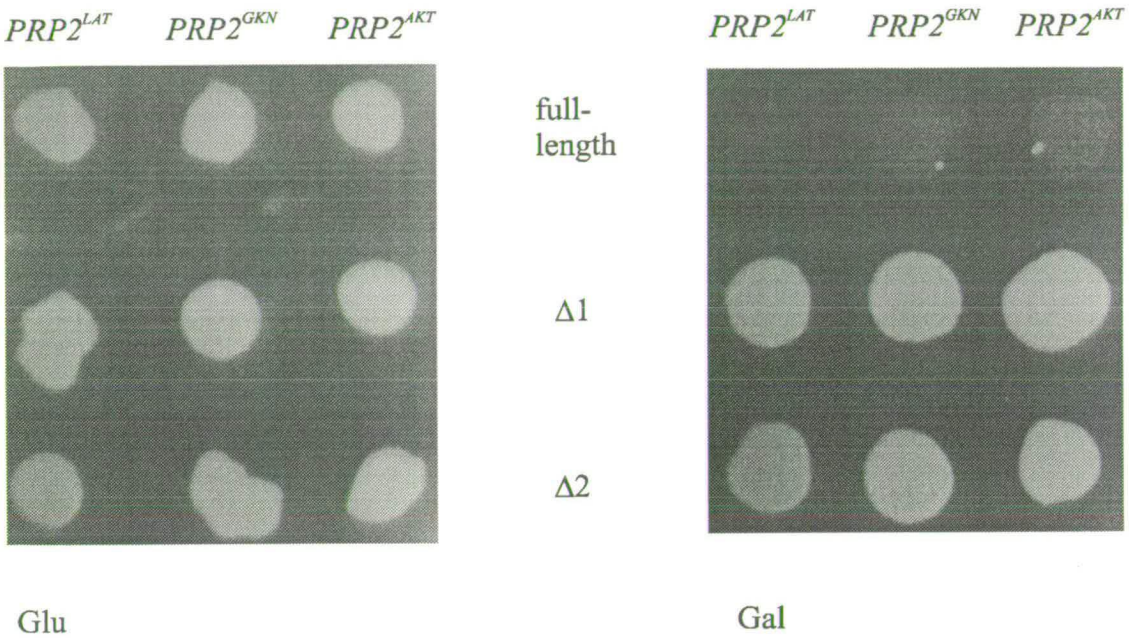


Figure 4.2. Purified transformants of S150 were suspended in sterile water and spotted onto selective glucose (Glu) and galactose (Gal) plates. Only the cells transformed with full-length dominant negative alleles are inhibited from growing in the presence of galactose at 30°C.

The full-length $Prp2^{AKT}$ protein was known to behave differently to the other dominant negative proteins, displaying dominance at or below 30°C (E. Flinn, 1994). The deleted forms of the dominant negative alleles were therefore tested at temperatures below 30°C. In the case of the $Prp2^{AKT\Delta1}$ protein, figure 4.3 shows that it retained dominance at 23°C and below. The $PRP2^{AKT\ \Delta2}$ mutation does not appear to have an effect on cell growth at any temperature.

Figure 4.3. Plate showing dominant negative effect of overexpressing *PRP2*$^{AKT\Delta1}$ at low temperatures.

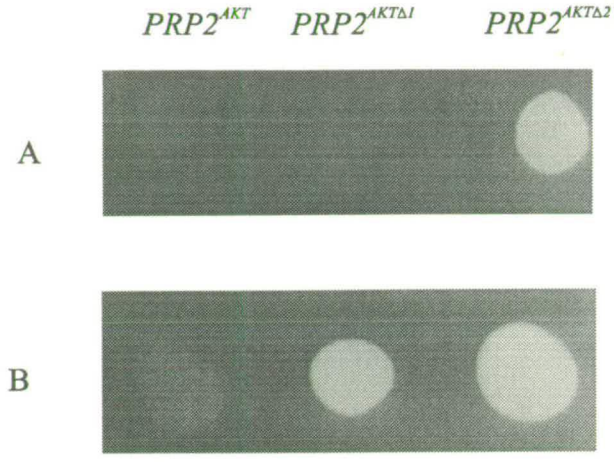


Figure 4.3. When grown in the presence of galactose at 23°C, pBMPRP2$^{AKT\Delta1}$ inhibits growth of transformants (A). At 30°C, only the full-length pBMPRP2^{AKT} is dominant (B)

Figure 4.4 Northern analysis of S150 overexpressing *PRP2*$^{AKT\Delta1}$ and *PRP2*$^{AKT\Delta2}$.

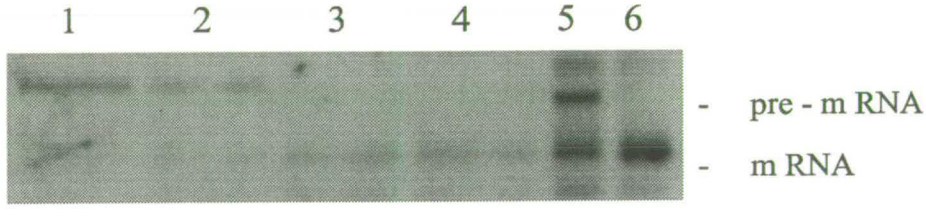


Figure 4.4 RNA was prepared from S150 transformed with pBMPRP2$^{AKT\Delta1}$ (tracks 1 and 3) pBMPRP2$^{AKT\Delta2}$ (tracks 2 and 4) or pBMPRP2^{AKT} (tracks 5 and 6). Tracks 1, 2 and 5 contain RNA from cells grown under inducing conditions (galactose). Tracks 3, 4 and 6 contain RNA from cells grown under repressing conditions (glucose). A northern blot of the RNA following electrophoresis was probed with labelled rp28.

However, northern analysis of RNA from cells grown in glycerol-lactate medium then induced by galactose shows cells transformed with either the *Prp2*$^{AKT\Delta1}$ and *Prp2*$^{AKT\Delta2}$ mutant alleles accumulate unspliced pre-mRNA *in vivo* (figure 4.4). This is not seen in the presence of the other mutant constructs.

Figure 4.5 Western blot showing overproduction of dominant negative alleles.

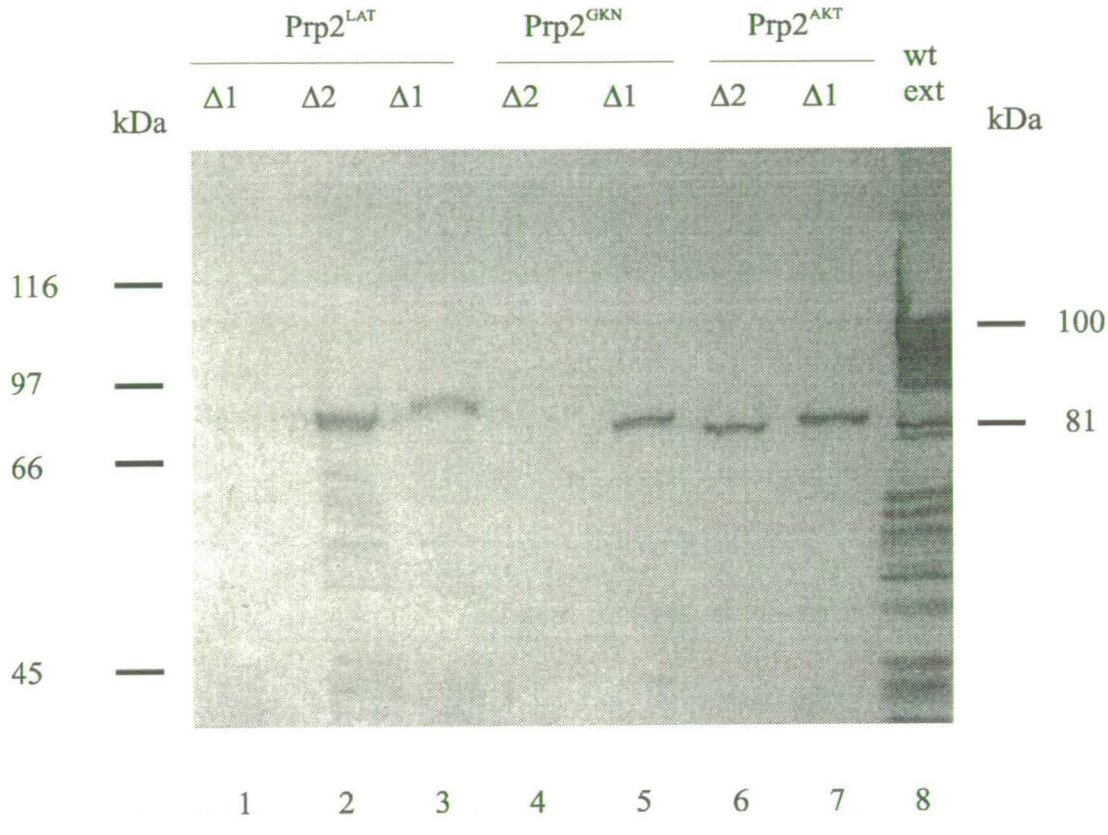


Figure 4.5. Equal amounts of crude protein extracts as determined by Bradford assay were separated by SDS-PAGE and electroblotted to Immobilon-P. Track 1 contains protein from uninduced cells, tracks 2-7 contain protein from cells overproducing shortened dominant negative Prp2 protein (81kDa), and track 8 contains extract overproducing full-length Prp2 protein (100kDa). Other western blots have shown $Prp2^{GKN}$ (track 4) to be overproduced.

Western analysis (figure 4.5) shows that where dominance is lost, as in the cases of the $PRP2^{GKN}$ and $PRP2^{LAT}$ mutant alleles, it is not due to degradation or lack of expression of the mutant protein.

4.2.2 *In vitro* characterisation of $PRP2^{AKT\Delta I}$.

Splicing extracts made from overexpressing $PRP2^{AKT\Delta I}$ in a wild-type background do not splice. When combined with different ratios of wild-type splicing extract, the splicing

activity of the wild-type extract can be blocked. Figure 4.6 shows the effect of adding overexpressing *PRP2*$^{AKT\Delta I}$ extract to a wild-type S150 extract.

Figure 4.6. Inhibition of splicing of S150 splicing extract by overexpressing *PRP2*$^{AKT\Delta I}$ splicing extract.

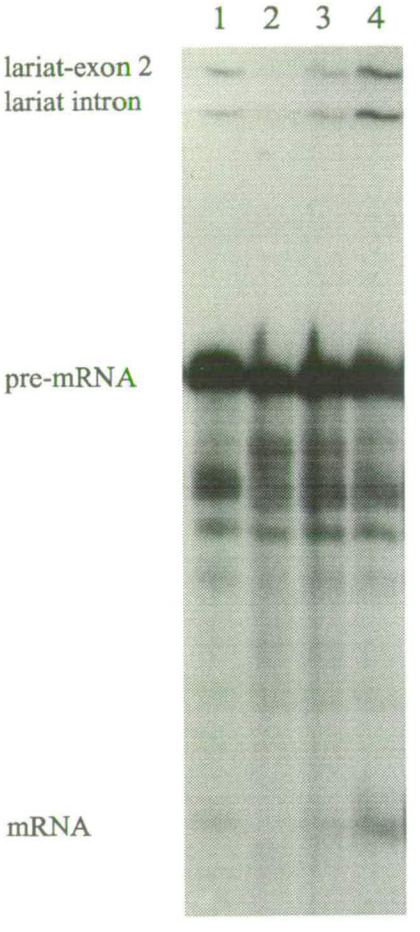


Figure 4.6. Splicing extract made from S150 carrying pBMPRP2$^{AKT\Delta I}$ induced by galactose was added to S150 splicing extract. Tracks contained the following amounts of each extract:

Track	S150 extract (µl)	S150/ pBMPRP2$^{AKT\Delta I}$ extract (µl)
1	2	-
2	2	4
3	2	3
4	2	2

Splicing activity of S150 extract is reduced by addition of S150/ pBMPRP2$^{AKT\Delta1}$ extract.

Native gel electrophoresis shows the complexes involved in splicing. In the wild type case, complex III is made first, which includes the U1 and U2 snRNPs assembled on the pre-mRNA. This is converted to complex I , and around the time of step one of splicing, complex I is converted to complex II. In extracts containing high levels of dominant negative protein, complexes III and I are seen, but complex II does not form and no splicing takes place.

Figure 4.7. Native gel showing splicing complexes formed by wild-type splicing extract and overexpressing *PRP2$^{AKT\Delta1}$* extract.

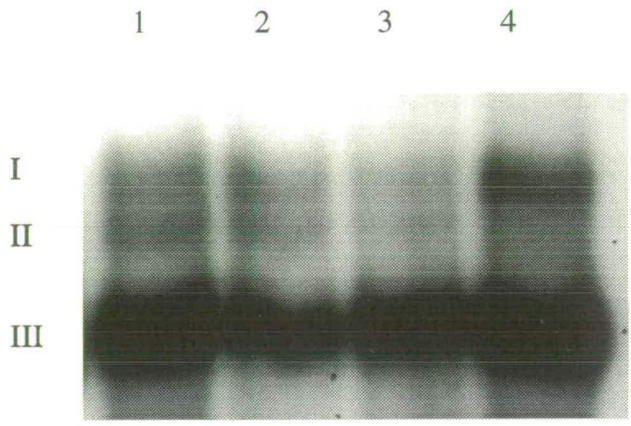


Figure 4.7. Tracks 1, 2 and 3 show formation of complexes I, II and III, 5, 10 and 15 minutes after starting a splicing reaction with S150 splicing extract. Track 4 shows formation of complexes I and III only by S150/ pBMPRP2$^{AKT\Delta1}$ extract after 15 minutes.

After native gel electrophoresis of splicing complexes produced in an overexpressing *PRP2$^{AKT\Delta1}$* extract, complexes III and I are resolved.

4.2.3 RNA binding.

The dominant Prp2^{AKT} mutant protein is known to have little or no ATP hydrolysis activity. This is not due to an inability to bind RNA however, as the Prp2^{AKT} mutant protein is shown to bind poly(U) (M. McGarvey, personal communication) during the

Prp2 protein purification procedure (Plumpton *et al*, 1994). It seems therefore that either RNA binding fails to stimulate ATP hydrolysis, or that this protein is unable to bind ATP. By analogy with yeast Ras2 protein, it is a possibility that $Prp2^{AKT}$ is locked in an inactive form which is unable to bind RNA (see section 1.11). Dominant negative mutants of Ras2 are known to have a higher affinity for GDP over GTP, which prevents the mutant protein being activated by nucleotide exchange (Powers *et al*, 1989).

As the $Prp2^{AKT}$ mutant protein retains some dominant negative activity in the Δ1 form, poly(U) binding experiments were carried out. Splicing extracts prepared from cells that overproduced $Prp2^{AKT\Delta1}$ or $Prp2^{AKT\Delta2}$ in a wild-type background were incubated with poly(U) agarose in a procedure modified from Kim *et al*, 1992.

1) 100mg poly(U) agarose in a microcentrifuge tube was swollen in 1M NaCl/50mM Tris pH 7.5 for 30 min with mixing at room temperature. The beads were then washed X5 with 1ml BP buffer/50mM KCl at 4°C (section 2.5.10.4).

2) After the last wash was removed, 1ml of the sample to be bound to poly(U) (usually splicing extract) was added to the beads and incubated at 4°C with gentle mixing for 2h.

3) The beads were pelleted and washed X10 with 1ml of 100mM KCl in BP at 4°C.

4) The bound RNA was eluted with X5 0.5ml of 150mM KCl in BP, followed by 5X 200mM KCl in BP and a final X5 with 250mM KCl in BP.

30µl aliquots of each eluted fraction were subjected to SDS-PAGE (section 2.5.2.2), western blotted to Immobilon P and probed with anti-Prp2 antibodies anti-55 (section 2.5.4).

Figure 4.8 RNA-binding by Prp2$^{AKT\Delta1}$.

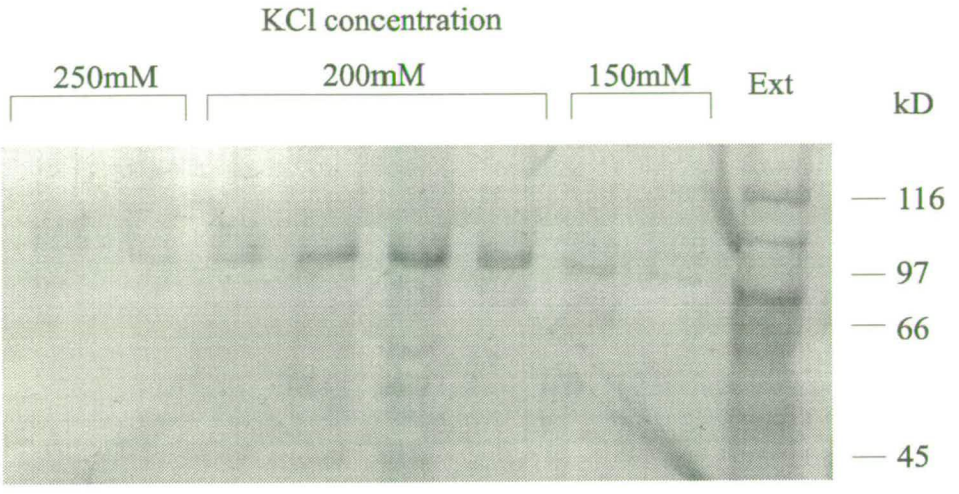


Figure 4.8. Splicing extract (Ext) made from overexpressing S150/pBMPRP2$^{AKT\Delta1}$ cells was incubated with poly(U) agarose as above. 30µl aliquots from each eluted fraction were subjected to SDS-PAGE and western blotted to Immobilon-P membrane which was probed with anti-55 antiserum. Most Prp2$^{AKT\Delta1}$ protein was eluted by 200mM KCl.

Figure 4.8 shows that the 81kDa Prp2$^{AKT\Delta1}$ protein binds to poly(U), eluting at a slightly lower concentration of salt (200mM KCl) compared to the full-length Prp2^{AKT} protein (250mM KCl; Plumpton *et al*, 1994 and data not shown). Similar results were obtained for Prp2$^{AKT\Delta2}$.

4.3 Discussion.

We have shown that in the case of one dominant-negative allele of *PRP2*, *PRP2*^{AKT}, some dominant negative activity is retained after deletion of certain regions from this protein. This was in contrast to similar deletions in other alleles that resulted in non-functional proteins. Additionally, it has been suggested that the dominant activity may be linked to the RNA binding activity of Prp2 (Plumpton *et al*, 1994, Teigelkamp *et al*, 1994). Teigelkamp *et al* showed the dominant negative *PRP2*^{LAT} mutant forms a stable association with the pre-messenger RNA in the spliceosome. Deletion or disruption of the RNA binding site within the Prp2 protein could cause the loss of dominant negative activity. The Δ1 deletion removes a likely RNA-binding site, the QRIGR motif (Pause

et al, 1993), and also removes half of the putative zinc finger motif. The Δ2 deletion removes the other part of the putative zinc finger sequence. Loss of dominance over growth is observed with all the deletions with the exception of $Prp2^{AKT\,\Delta1}$.

In the case of many RNA binding proteins, there are several domains in the one protein which are capable of binding RNA. These domains can work in *cis* (Shamoo, *et al*, 1994) or in *trans* (Lutz-Freyermuth, 1990), binding either one or several RNAs. The deletion of one or more RNA binding domain can result in decreased affinity or specificity of RNA binding in some proteins, whereas other proteins require all their RNA binding domains to function. The loss or disruption of these putative RNA binding domains of Prp2 may account for the loss of dominance.

There are several differences between the $PRP2^{AKT}$ mutant and other dominant negative alleles of *PRP2*. The most striking difference is that $PRP2^{AKT}$ mutant exhibits dominance only at 30°C and lower temperatures, whereas the other dominant negative mutants of *PRP2* are dominant at all temperatures. Biochemical studies of purified proteins have shown that in contrast to $Prp2^{LAT}$, $Prp2^{AKT}$ protein has little or no ATP hydrolysis activity. $Prp2^{GKN}$ has similarly a negligible rate of ATP hydrolysis but in this case, it is likely to be due to lack of stimulation by RNA binding, as $Prp2^{GKN}$ does not bind poly(U). Immunoprecipitation experiments have failed to show any association of $Prp2^{AKT}$ with the spliceosome in contrast to the stable association formed between the spliceosome and $Prp2^{GKN}$ or $Prp2^{LAT}$ (E. Flinn, 1994, Plumpton *et al*, 1994).

In the light of these differences, it is interesting that $Prp2^{AKT}$ is the one mutant protein which retains some dominant phenotype after deletion of the *Xba*I to *Pvu*II (Δ1) fragment. Both the $Prp2^{LAT}$ and $Prp2^{GKN}$ proteins are thought to be dominant negative by means of a stable association with the spliceosome which has been demonstrated by immunoprecipitation experiments (E. Flinn, 1994, Plumpton *et al*, 1994). This association has not been shown in the case of the $Prp2^{AKT}$ protein, although unlike $Prp2^{GKN}$, $Prp2^{AKT}$ does bind poly(U). The fact that the Δ1 deletion does not fully inactivate the $Prp2^{AKT}$ protein suggests that all the activity of this protein is not lost but an important functional domain is removed. Both the $Prp2^{AKT\Delta1}$ and $Prp2^{AKT\Delta2}$ proteins

retain the ability to bind RNA, so not all function of these proteins is lost. If the dominant negative activity of $Prp2^{AKT}$ is not due to formation of a stable association with the spliceosome, then removal of the spliceosome binding domain would not abrogate dominant function in this protein. Taken together, these results suggest that $Prp2^{AKT}$ protein confers its dominant effect in a different way to the other dominant negative mutants, perhaps by sequestering some other factor which interacts with the Prp2 protein.

Figure 4.9. Model of proposed action of $Prp2^{AKT}$ protein.

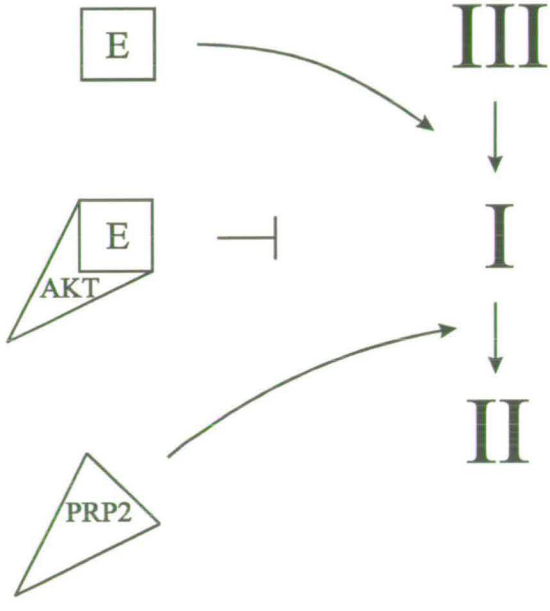


Figure 4.9. Factor E is required for complex III-I transition. $Prp2^{AKT}$ protein has a high affinity to factor E and can bind. preventing the normal function of E. $Prp2^{AKT}$ in the E-bound form is unable to enter preformed spliceosomes. $Prp2^{WT}$ protein interacts transiently at the complex I-II transition, but this function is overridden by the action of $Prp2^{AKT}$.

A model was first proposed by E.Flinn (1994) and is summarised in figure 4.9, in which $Prp2^{AKT}$ protein binds to a factor involved in complex III to I transition. This was suggested by the finding that partially purified $Prp2^{AKT}$ protein inhibits the formation of complex I when added to wild-type splicing extracts, although extracts made from overexpressing $Prp2^{AKT}$ in a wild-type background allow the formation of both complexes III and I.

The model suggests that $Prp2^{AKT}$ sequesters a factor in splicing extracts with the result that complex III to I transition does not take place. On the basis of this model, it is conceivable that deleted forms of $Prp2^{AKT}$ retain some affinity for putative factor E. A candidate for factor E is a nucleotide exchange factor (NEF) for Prp2. If $Prp2^{AKT}$ exerts dominant negative activity by sequestering a putative NEF, the domain required for this activity could still be present and to some extent functional in $Prp2^{AKT\Delta I}$.

Chapter five.

A Prp2-depleted *in vitro* system.

5.1 Introduction.

The advent of a simple and efficient system for purification of Prp2 protein (Plumpton *et al*, 1994) enables many *in vitro* studies which are informative of the effect of mutations on the activity of the Prp2 protein. To assay the effect on splicing, complex formation, spliceosome binding and pre-mRNA binding, an *in vitro* system derived from a heat-inactivated *prp2-1* mutant splicing extract is currently used. A point mutation in *prp2-1* causes a temperature-sensitive defect. Splicing extracts can be made from a strain carrying this mutation grown at the permissive temperature. The extracts can then be heat-inactivated *in vitro* by incubating at 36°C for one hour. Spliceosomes can be assembled and purified from these extracts (section 2.7.8) which do not go through splicing, as the Prp2 protein necessary for step 1 is present in a heat-inactivated form. However, this system assumes that Prp2 protein has only one function which is heat-sensitive in the mutant protein, and that the heat-inactivated protein does not interfere with any subsequent assays such as immunoprecipitation experiments to show association with the spliceosome.

Results previously presented (E. Flinn, 1994; this thesis, chapter four) suggest that, potentially, Prp2 protein may have another role in splicing prior to the transient interaction with the spliceosome seen at step 1, which could explain the dominant activity of the $Prp2^{AKT\Delta1}$ protein. This may be an interaction with another factor, possibly involved in formation of complex I. It is conceivable that the heat-inactivated prp2-1 protein retains such a function early in splicing and is only inactivated at the step 1 stage. To investigate this possibility, an attempt was made to develop an *in vitro* system entirely lacking any form of Prp2 protein.

Immunodepletion with anti-Prp2 antibodies was previously used in an attempt to create a totally Prp2-free system but with this approach it as difficult to remove sufficient Prp2 protein, even when using a combination of different anti-Prp2 antibodies, preventing

this from becoming a viable strategy (M. Lossky, 1988). One of the problems encountered using immunodepletion was the low efficiency of precipitation by anti-Prp2 antibodies. A relatively large volume of antibodies (40µl) had to be bound to 20-30µl PAS to deplete 10µl of splicing extract of Prp2 resulting in dilution of the extract and resultant loss of activity. An attempt to compensate for this dilution by increasing the relative volume of splicing extract in a splicing reaction failed to increase the level of splicing accordingly.

Another problem with the antibody system was the long (3-4h) incubation of splicing extract/PAS-anti-Prp2 at 4°C. Extract incubated alone at this temperature for this length of time also showed a marked decrease in splicing compared to freshly-thawed extract.

Several adjustments were made by Lossky in order to overcome these problems. It was found that when extracts were not irreversibly inactivated by the immunodepletion process, some splicing activity remained. This can be explained by the finding that heat-inactivated *prp2-1* splicing extract can be complemented by a 40-fold dilution of splicing extract wild-type for *PRP2* (Lin *et al*, 1987), suggesting that *in vitro* at least, Prp2 protein is present in excess.

Where extracts were irreversibly inactivated, it is a possibility that other essential splicing factors cross-react with the anti-Prp2 antibodies and are also depleted. Given the similarity between Prp2, Prp16 and Prp22 proteins (Chen and Lin, 1990), this is entirely possible.

A new strategy was developed in order to take advantage of the His-tagging system. Depletion of Prp2 protein from splicing extracts by Ni-NTA resin should overcome some of the problems presented by immunodepletion. The Ni-NTA resin has a higher binding affinity to $Prp2^{HIS}$ relative to that of anti-Prp2 antibodies. Using this system, it should be possible to deplete a splicing extract of Prp2 without the dilution problems presented by immunodepletion. The length of incubation at 4°C can be decreased relative to immunodepletion, reducing the possibility of loss of activity.

5.2 Results.

5.2.1 Construction of SAy2 and SAy3.

In order to minimise the amount of Ni-NTA resin necessary for depletion of Prp2, and therefore avoid a large dilution effect, a strain in which the expression of *PRP2* could be regulated was constructed. This was carried out in two stages.

The diploid strain EFy1 (**a/α**, *ura3-52/ura3-52, his3-Δ200/his3Δ1, ade2-101oc/ADE2, lys2-801/LYS2, trp1-Δ1/trp1-289, LEU2/leu2-3,112, PRP2/PRP2::HIS3*) was transformed with the constitutive *PRP2* plasmid pSA10 (*PRP2*, *TRP1*) and transformants selected on minimal medium lacking tryptophan. Transformants were tested for the appropriate markers and sporulated. Tetrads were dissected as previously described and the progeny from each tetrad tested for markers. A strain was purified from one of the progeny which upon testing was found to have the phenotype His^+, Trp^+, ade^- and ura^-. This strain was called SAy2.

A plasmid shuffle was then performed on SAy2 to obtain the strain SAy3. SAy2 was transformed with $pBMPRP2^{HIS}$ (galactose-inducible *PRP2, URA3*) and transformants selected for the ability to grow on galactose plates lacking uracil. Cells were then grown to stationary phase twice in drop-out liquid media maintaining the selection for the $pBMPRP2^{HIS}$ plasmid and containing galactose to induce expression of *PRP2*, prior to plating out on similar agar. Colonies were tested for the absence of pSA10 and the presence of $pBMPRP2^{HIS}$. One such strain isolated was called SAy3.

5.2.2. *In vivo* characterisation of SAy3.

Growth curves of SAy3 in glucose and galactose media were obtained. A single colony was picked into YPGRA medium. Mid-log cells were pelleted and resuspended in either YPDA or YPGRA then used to inoculate pre-warmed and pre-aerated YPDA or fresh YPGRA. OD_{600} was determined over a 50 hour period, and to maintain logarithmic growth, the cells were diluted into fresh pre-warmed and pre-aerated media whenever the OD_{600} approached 0.5. Complete medium was used, as selection pressure

for the plasmid is maintained due to the sole copy of *PRP2* being plasmid-borne. 20ml of culture were removed at every timepoint and used to make crude protein extracts. Figure 5.1 clearly shows that while growth continues exponentially in the presence of galactose, the growth rate of cells in glucose medium slows down 16 hours after switching from galactose. The *GAL1* promoter does however, allow a low level of expression in the presence of glucose which allows the cells to continue growing, although at a much reduced rate. This is confirmed by western analysis (figure 5.2) which shows a dramatic decrease in the level of production of Prp2 protein after around four hours growth in glucose although a small amount of Prp2 protein is detectable even after 50 hours in glucose.

5.2.3. Prp2-depletion of splicing extracts with Ni^{2+} resin.

SAy3 was grown to OD_{600} 0.5 in YMGlyLac in which the *GAL1* promoter is neither induced nor repressed, then grown in the presence of 2% glucose for four hours before harvesting. Splicing extracts were prepared from cells grown in this way. Cultures were incubated at 23°C at all times.

Figure 5.1 Growth Curve of SAy3 in Glucose and Galactose

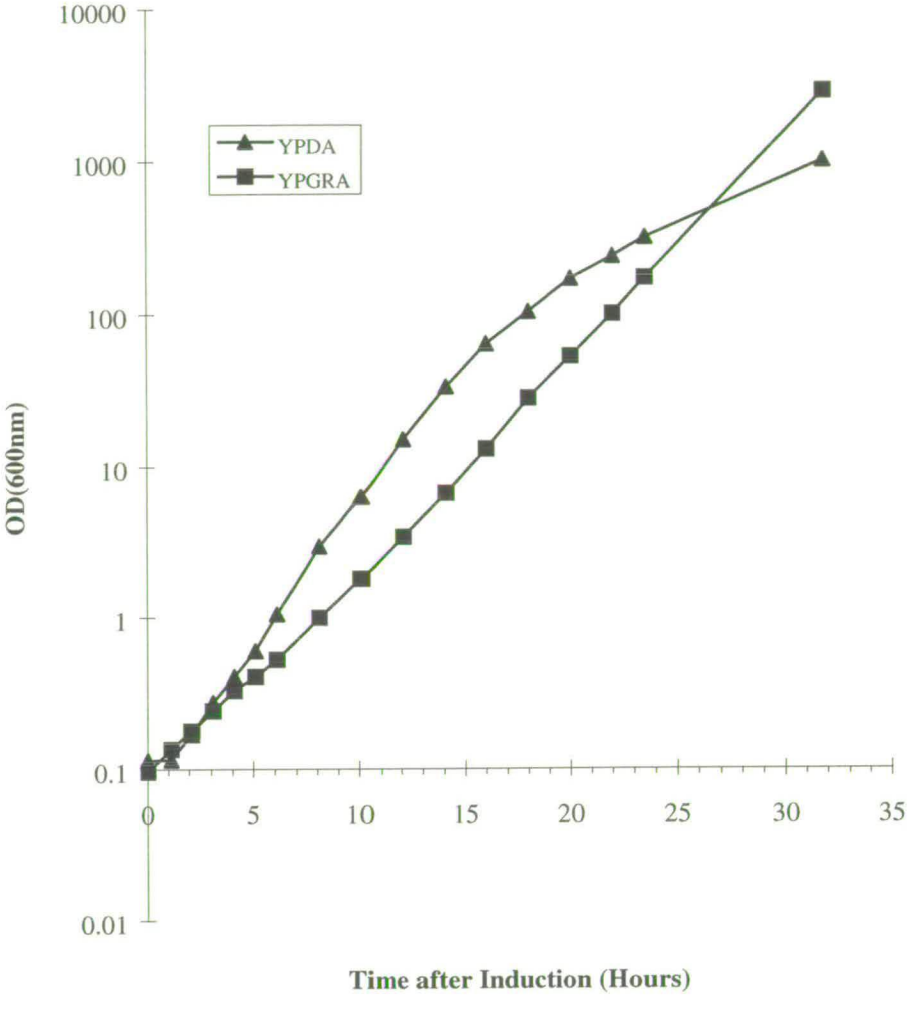


Figure 5.1. SAy3 cells were grown in glucose (YPDA) or galactose (YPGRA) medium. OD_{600} was measured at regular intervals and plotted against time to generate the above growth curve.

Figure 5.2. Western showing decreasing levels of production of Prp2 protein with increasing time of incubation in glucose medium.

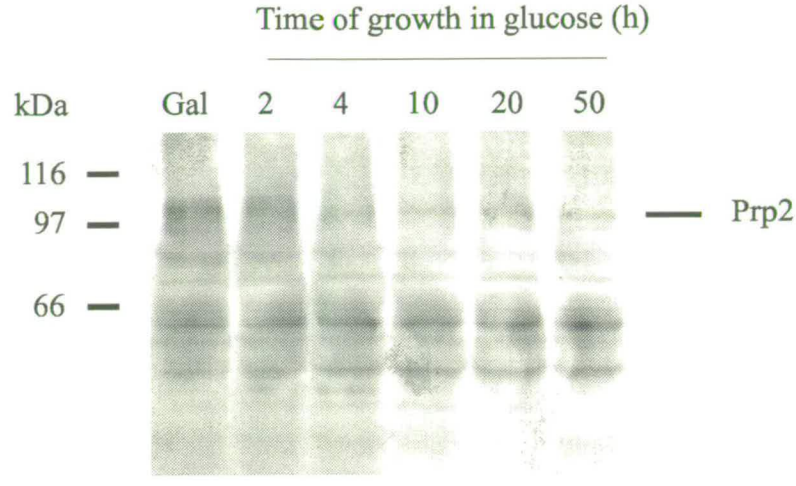


Figure 5.2. Equal amounts of crude protein extracts as determined by Bradford assay were analysed by Western blotting. This shows that four hours incubation in glucose is enough to significantly reduce the amount of Prp2 protein present. Prp2 is still detectable after 50 hours growth in glucose.

Splicing extracts were further depleted of Prp2 protein by Ni-NTA resin in a batch procedure modified from the column purification protocol of Plumpton *et al*, 1994.

1) 40µl (packed volume) Ni-NTA resin in a microcentrifuge tube was washed 5X with prewash solution at 4°C (see section 2.5.10.3 for all solutions).

2) 500µl 2X sample buffer (section 2.5.10.3) was added to 500µl SAy3 splicing extract and imidazole added to a final concentration of 1mM. Splicing extract and sample buffer were added to the washed Ni-NTA resin and incubated at 4°C for 1h with gentle mixing.

3) The Ni-NTA resin was pelleted and the splicing extract removed and dialysed for 3h against buffer D at 4°C.

5.2.4 *In vitro* analysis of depleted SAy3 splicing extracts.

SAy3 extract treated as above was used in splicing reactions. Figure 5.3 shows that although splicing is abrogated following the depletion process, activity cannot be regained by the addition of purified Prp2 protein alone, or by the addition of purified prp2 protein and heat-inactivated DJy85 (*prp2-1*) splicing extract.

Figure 5.3. In vitro splicing by SAy3 extract before and after depletion by Ni-NTA resin.

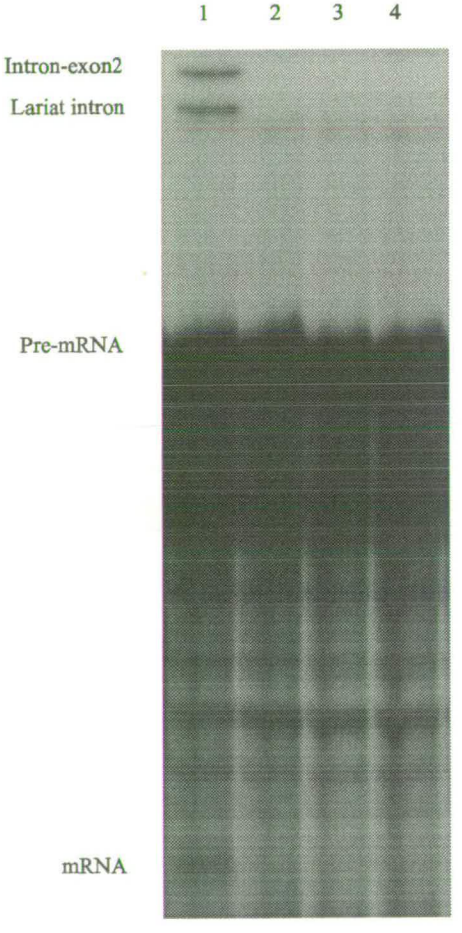


Figure 5.3. Splicing of rp28 transcript by 1:SAy3, 2:SAy3depleted by Ni-NTA resin, 3: as track 2 but with the addition of purified $Prp2^{HIS}$ protein, 4: as track 3 but with the addition of heat-inactivated DJy85 (*prp2-1*) extract.

However, as the splicing activity of this extract before depletion was low, having around 1/10 activity compared to wild-type extracts, experiments to investigate the abrogation

of splicing by the depletion process were carried out using wild-type splicing extracts. Adding 2X sample buffer to wild-type splicing extract was subsequently shown to abolish splicing activity even after dialysis of the extract into buffer D (figure 5.4).

Figure 5.4. Splicing is inhibited by the addition of 2X sample buffer.

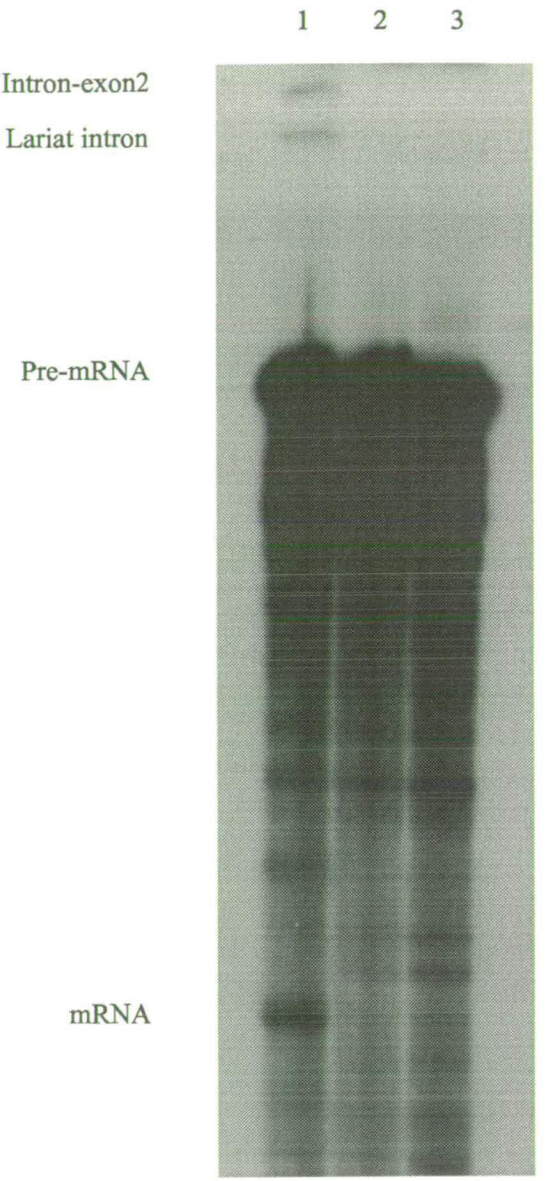


Figure 5.4. Splicing activity of S150 extract (1) is abrogated by the addition of 2X sample buffer (2). Following dialysis of extract treated with 2X sample buffer into buffer D, splicing activity is not regained (3).

Comparison of 2X sample buffer with splicing buffer A shows a vast difference in salt concentration (table 5.1).

Table 5.1 Comparison of 2X sample buffer and splicing buffer A.

2X Sample Buffer	Splicing Buffer A
20mM Tris pH7.9 20% glycerol 1M NaCl 2mM imidazole pH7.9	10mM HEPES pH7.9 1.5mM $MgCl_2$ 10mM KCl

It is conceivable that the high salt concentration disrupts protein/protein or protein RNA interactions in the splicing extract which are unable to reform *in vitro* when the salt has been removed by dialysis.

5.2.5 Development of splicing-compatible depletion system.

It was found that the addition of 2X sample buffer to a wild-type splicing extract abolished splicing (figure 5.4). If the Prp2 protein could be depleted from the splicing extract without the addition of buffers detrimental to splicing, it may facilitate the restoration of splicing activity in such extracts.

To determine the binding efficiency of Ni-NTA resin to Prp2 protein in splicing buffer, Prp2 protein was purified from a splicing extract in which Prp2 has been overproduced in a wild-type background. Small-scale purification was carried out in a batch procedure with modified buffers as follows.

1) 400µl Ni-NTA resin was equilibrated with splicing buffer A (section 2.5.6.1) by washing X5 with 1ml.

2) 0.5-1ml splicing extract was added to the prepared Ni-NTA resin and incubated at 4°C for 1h.

3) The beads were pelleted and the splicing extract removed.

4) The beads were washed X5 with 1ml SCE buffer (see below) containing 50mM imidazole, followed by 3X 1ml washes with 150mM imidazole in SCE.

5) Protein was eluted with 5X 0.5ml 200mM imidazole in SCE.

SCE buffer (Splicing-Compatible Elution) was based on buffer D used during preparation of splicing extracts (section 2.5.6.1) and is composed of 20mM HEPES pH7.5, 100mM KCl and 20% glycerol.

Western analysis of wash and elution fractions (figure 5.5) shows that Prp2 protein binds to the Ni-NTA resin efficiently under these conditions and is eluted at the same imidazole concentration (250mM) as during conventional purification (Plumpton *et al*, 1994).

Figure 5.5 Western analysis of fractions from batch purification of Prp2^{HIS} protein.

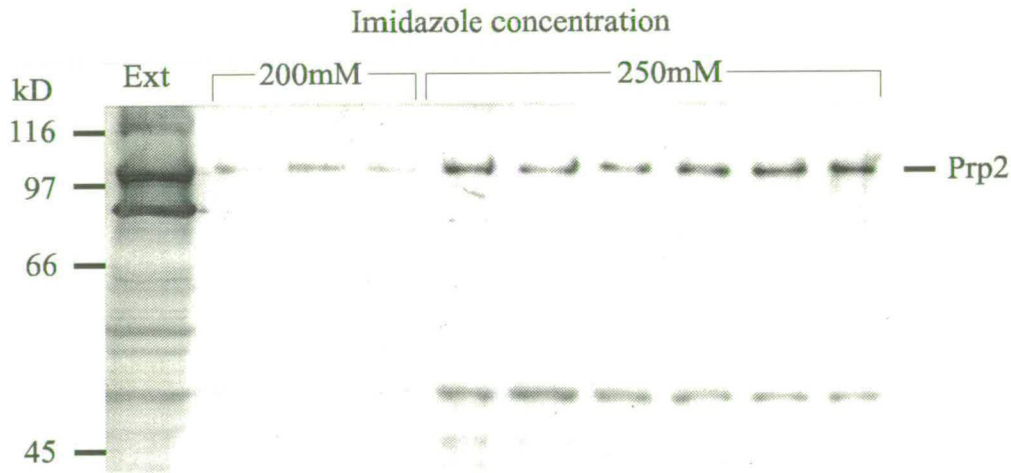


Figure 5.5. Western analysis of 30µl aliquots from elution fractions shows that most Prp2 protein is eluted by 250mM Imidazole.

Although the above system is efficient in the purification of Prp2^{HIS} protein, S150/pBMPRP2^{HIS} splicing extract treated in this way had greatly reduced splicing activity.

5.3 Discussion.

A strain has been created in which it is possible to reduce the level of expression of *PRP2* to slightly below that of wild-type by repression of the *GAL1* promoter by growth in the presence of glucose. Production of Prp2 protein in this strain, SAy3, is reduced but not abolished, even when grown in the presence of glucose for over 50 hours.

Splicing extracts made from this strain grown in the presence of glucose splice weakly if at all, even after minimal (4h) exposure to glucose. Splicing in these extracts cannot be augmented or complemented by the addition of purified Prp2 protein, suggesting that depletion of Prp2 protein *in vivo* may result in the depletion of one or more other factors essential for splicing. The *in vitro* depletion process may also remove factors essential for splicing. Other proteins are eluted from the Ni-NTA resin in fractions containing Prp2 protein. It is possible that one of these proteins is essential for splicing activity. It is not inconceivable that the level of exression of PRP2 is limiting when Say3 is grown in glycerol-lactate medium in the absence of inducing galactose.

Even if a depletion system is developed which does not irreversibly inactivate a splicing extract, there is a possibility that splicing activity will not be completely abolished. Only low levels of Prp2 protein are required for splicing *in vitro* (Lin *et al*, 1987). There is a possibility that although $Prp2^{HIS}$ protein is efficiently purified by the Ni-NTA resin, a residual amount of Prp2 may be sequestered in some way at the time of depletion so as to make the poly-histidine tag inaccessible. This would result in a low level of Prp2 protein being present after the majority of Prp2 protein has been removed.

An approach which could combine the efficiency of the Ni-NTA system with the specificity of the antibody depletion would be to have an epitope-tagged Prp2 expressed constitutively at low level which can be removed from splicing extracts using monoclonal antibodies. This approach may facilitate the removal of all the Prp2 protein without the detrimental effects of glucose repression and non-specific removal of other splicing factors.

Chapter six.

The role of *PRP2* in the fidelity of splicing.

6.1 Introduction.

As stated earlier in this thesis, Prp2, Prp16 and Prp22 proteins are members of the DEAH-box subfamily of RNA-dependent ATPases. *PRP16* was first isolated as a mutant, *prp16-1*, which suppressed an A to C mutation of the branch nucleotide.

The *prp16-1* suppressor mutation is contained in the highly conserved central domain. A mutation of a single conserved amino acid residue near the ATP-binding site results in a tyrosine to asparagine change in amino acid position 386 of the Prp16 protein.

In vivo characterisation of the *prp16-1* mutation revealed the presence of a growth defect. Cells carrying the *prp16-1* mutant allele grow slower at low temperatures (18°C and 23°C). This growth defect is dominant and exacerbated by overexpression of the mutant allele, which results in a reduction in growth rate by a factor of 2.2 at 18°C (Schwer and Guthrie, 1992).

In vitro characterisation has shown that *prp16-1* causes accumulation of actin pre-mRNA and lariat-exon 2 splicing intermediate RNAs (Couto *et al*, 1987).

Purification of the Prp16 wild-type and mutant proteins revealed that the Prp16-1 protein had a reduced ATP hydrolysis activity, such that the mutant protein hydrolyses ATP at around 10% that of the wild-type rate.

Purified prp16-1 protein has been reported to form a stable association with Prp16-depleted spliceosomes blocked at step 2 in the presence of ATP (Schwer and Guthrie, 1991).

Due to the similarity between Prp2 and Prp16 proteins, and the proposal that Prp16 plays a role in proofreading the branch nucleotide, it was decided to investigate a

possible role for Prp2 protein in the fidelity of splicing by creating a mutation in *PRP2* analogous to the *prp16-1* mutation.

6.2 Results

6.2.1 Construction of *prp2-9*.

A T to G change was introduced at nucleotide 978 of *PRP2*, in pTZPRP2 by the Kunkel method of site-directed mutagenesis. This caused a Tyr to Asp change in the Prp2 protein sequence equivalent to that of prp16-1 protein. The entire *Hin*dIII-*Xba*I fragment of a mutant pTZPRP2 was sequenced to check for other mutations, then subcloned into *Hin*dIII-*Xba*I cut pSA3.

Figure 6.1. Complementation of DJy36 (*prp2-1*) by pBMPRP2-9.

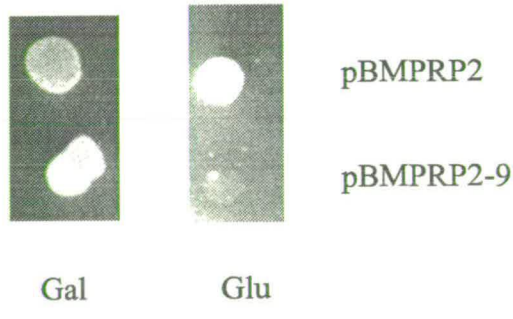


Figure 6.1. DJy36 cells transformed with either pBMPRP2 or pBMPRP2-9 were suspended in sterile water and spotted onto glucose (Glu) or galactose (Gal) plates and incubated at 36°C. pBMPRP2-9 is unable to complement the ts defect of the *prp2-1* cells on glucose but can complement on galactose plates.

This galactose-inducible construct, pBMprp2-9, was transformed into the temperature-sensitive *prp2-1* strain DJy36 and tested on glucose and galactose plates for complementation. Figure 6.1 shows that pBMprp2-9 is able to complement the ts growth defect at 36°C under inducing conditions on galactose, but not under repressing conditions on glucose.

6.2.2 *prp2-9* supports growth as sole source of *PRP2* when overexpressed.

Complementation assays using the temperature-sensitive *prp2-1* strain DJy36 do not always reveal how functional a mutant protein is. Some zinc finger mutants can support growth when they are the sole source of *PRP2* in the cell yet cannot complement a *prp2-1* ts defect (section 3.2.11). For this reason, pBMprp2-9 was transformed into the disrupted diploid strain EFy1. Transformants were selected on minimal galactose medium lacking uracil.

After sporulation, random spores were tested for auxotrophic markers and some found to be His^+, indicating the presence of the disrupted genomic *Prp2::HIS3*, and Ura^+, indicating the presence of the pBMprp2-9 plasmid. His^-, Ura^+ spores were also found.

Figure 6.2. Complementation assay of EFy1 progeny transformed with pBMprp2-9.

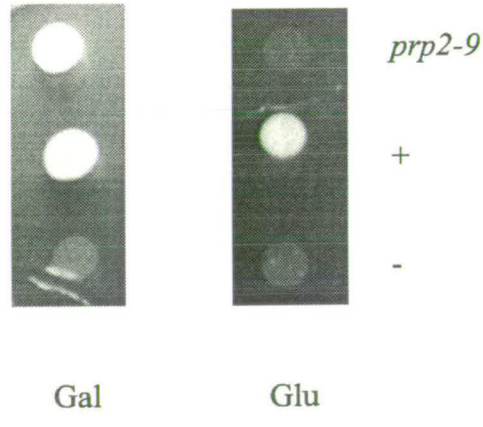


Figure 6.2. Haploid EFy1 progeny carrying a disrupted prp2 locus can grow on galactose (Gal) at 36°C but not on glucose (Glu).

Several isolates of His^+, Ura^+ progeny were plated on selective glucose and galactose medium and incubated at 36°C. Figure 6.2 shows that pBMprp2-9 is capable of supporting growth at the restrictive temperature, 36°C, on galactose but not on glucose.

This indicates that the prp2-9 protein has reduced efficiency compared to the wild-type Prp2 protein which can complement even during growth on glucose.

6.2.3 Can *prp2-9* support growth when expressed from the *PRP2* promoter?

As pBMprp2-9 is unable to complement the ts defect of *prp2-1* cells on glucose, a plasmid was constructed with the *prp2-9* mutant allele under the control of the *PRP2* promoter region. This was carried out over four steps (figure 6.3).

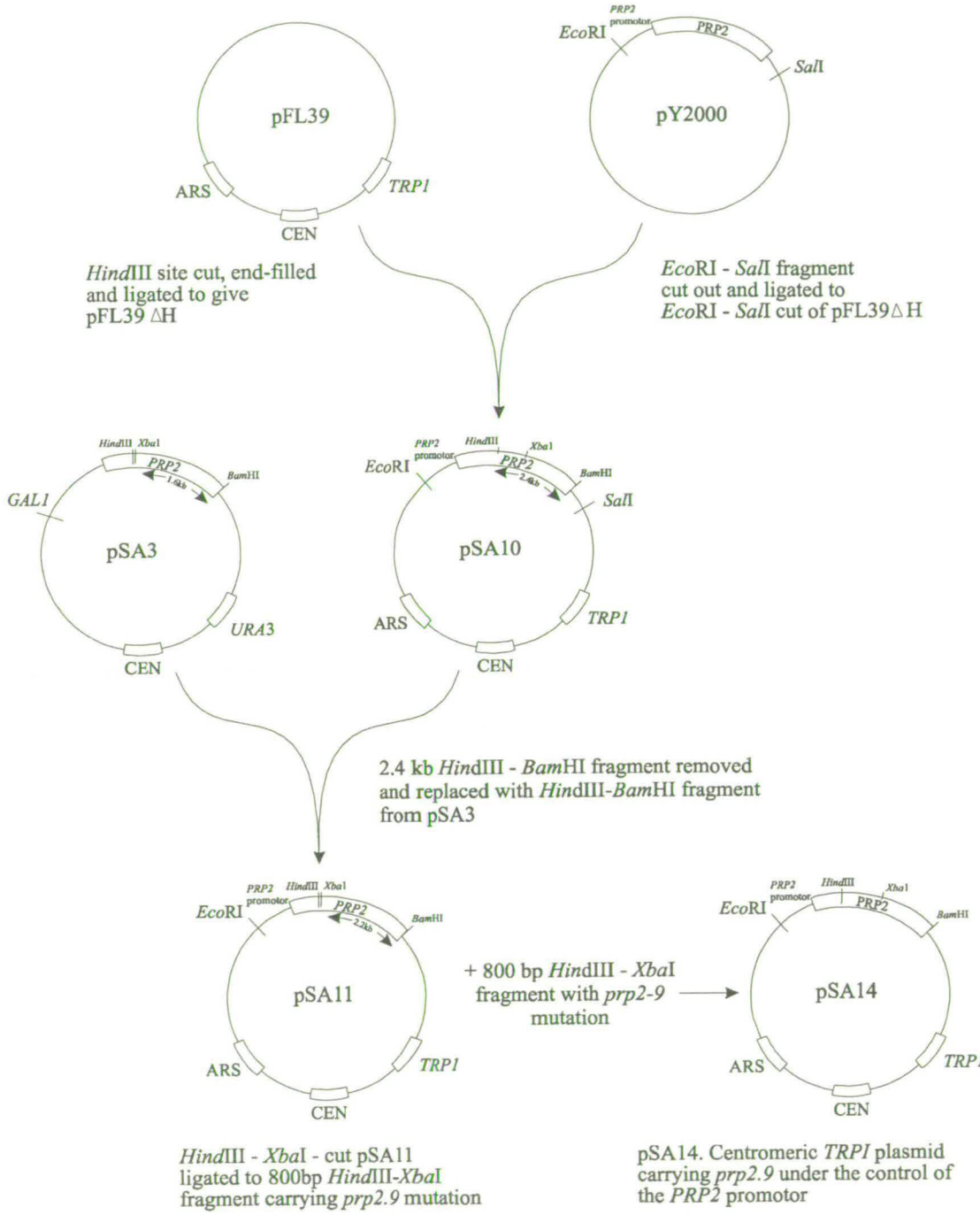
1) pFL39 (*ARS*, *CEN*, *TRP1*) was first cut with *Hin*dIII, the ends filled-in using Klenow fragment, and ligated to form pFL39ΔH. The cutting and end-filling of the *Hin*dIII site results in its destruction.

2) The *Eco*RI-*Sal*I fragment from pY2000, which includes the entire *PRP2* gene and the promoter region, was ligated into *Eco*RI-*Sal*I cut pFL39ΔH. This construct is known as pSA10.

3) This intermediate construct, pSA10, was cut with *Hin*dIII and *Bam*HI and the 2.4kb fragment replaced with *Hin*dIII-*Bam*HI fragment from pSA3 thus replacing the *Hin*dIII-*Xba*I sequence of *PRP2* with the *Sac*I linker sequence. This construct containing the *Hin*dIII-*Xba*I linker is known as pSA11.

4) An 800bp *Hin*dIII-*Xba*I fragment carrying the *prp2-9* mutation was ligated into pSA11 cut with *Hin*dIII and *Xba*I . The resulting *TRP1*/*prp2-9* construct under the control of the *PRP2* promoter is known as pSA14. pSA14 was transformed into EFy1

Figure 6.3. Construction of a centromeric *TRP1* plasmid with *prp2-9* under the control of the *PRP2* promoter.



which was then sporulated. Random spores were picked and screened for the appropriate markers. Cells from haploid strains which contained a disrupted genomic *prp2::HIS3* and carried the plasmid pSA14 (His^+, Trp^+) were resuspended in sterile water and spotted onto glucose plates lacking tryptophan and grown at 23°C and 36°C.

The strains tested grew at all temperatures. An example of one isolate growing at 36°C is shown in figure 6.4.

Figure 6.4. *prp2-9* supports growth when expressed from the *PRP2* promoter.

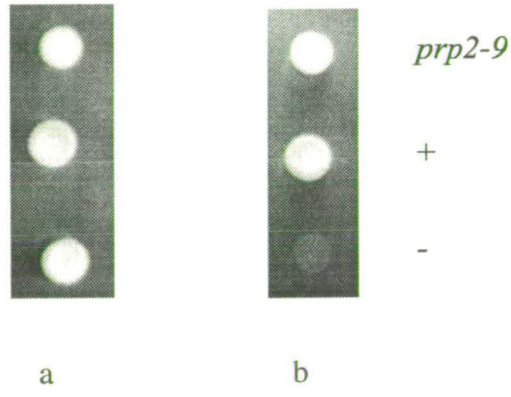


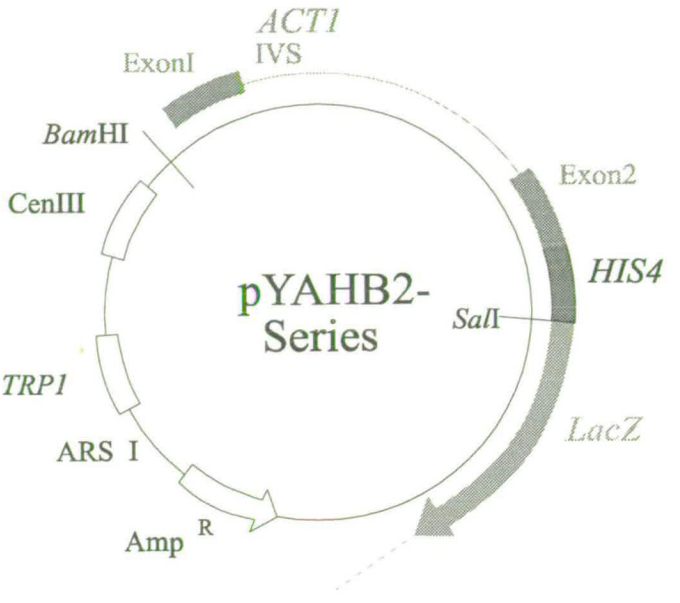
Figure 6.4. Haploid progeny from EFy1 transformed by pSA14 which carry the disrupted genomic *PRP2* locus grow on glucose plates at 23°C (a) and 36°C (b).

These results indicate that although *prp2-9* supports growth when expressed at a similar level to wild-type genomic *PRP2*, the reduced level of expression from the repressed *GAL1* promoter on glucose is not enough to support growth, suggesting that the efficiency of the prp2-9 protein is reduced compared to wild-type Prp2 protein.

6.2.4 β-galactosidase assays.

Two of the series of tester constructs used to characterise the *prp16-1* mutation were obtained (Couto *et al*, 1987). These plasmids contain the *ACT1* gene fused to lacZ (figure 6.5).

Figure 6.5. pYAHB2- series plasmids.



When the *ACT1* intron is correctly spliced, *lac*Z is expressed and can be measured using the *in vitro* assay for β-galactosidase activity as detailed in section 2.5.11. The level of β-galactosidase activity is limited by the level of intron splicing and can therefore be used as an indicator of splicing activity.

The constructs obtained contained the wild-type *ACT1* intron or with the G-A mutation at position 1 of the 5' splice site. Three additional mutations were made by site-directed mutagenesis. Intron mutants are summarised in Table 6.1.

The 1.4kb *Bam*HI-*Sal*I fragment of pYAHB2wt was ligated into *Bam*HI-*Sal*I cut pTZ19 to give pSA7. This was used as a template for the Kunkel method of site-directed mutagenesis. The resultant mutants were sequenced throughout the entire 1.4kb fragment before being ligated into *Bam*HI-*Sal*I cut pYAHB2wt. The identity of recombinant plasmids was confirmed by β-galactosidase assays as the relative activities for these mutants are known (Couto *et al*, 1987).

Table 6.1. Mutations in the 5' splice site and branchpoint sequences of *ACT1* intron in pYAHB2-series plasmids.

Plasmid	5' splice site	Branchpoint
pYAHB2wt	GUAUGU	UACUAAC
" A1	**A**UAUGU	UACUAAC
" C1	**C**UAUGU	UACUAAC
" A5	GUAU**A**U	UACUAAC
" C259	GUAUGU	UACUA**C**C

Intron mutations are shown in bold.

The reporter plasmids were co-transformed into S150-2B with the *PRP2* or *PRP16* allele to be tested on a different plasmid. The co-transformants were selected on minimal medium lacking uracil and tryptophan.

The two mutations in position one of the 5' splice site which almost completely abrogate splicing are not suppressed by any allele tested, giving negligible activity in any background. The G5 to A mutation, also in the 5' splice site, gives around 45% splicing, relative to the activity of the wild-type construct, regardless of background. In the case of the branchpoint mutation, *prp16-1* increases the level of splicing from a background of 8% to 23%, and the alleles of *prp2* also seem to suppress this mutation, giving from 18% to 24% activity. On glucose, the level of activity of all of these is between 5% and 8%, with only the constitutive *prp16-1* showing suppressor activity. These results are summarised in Table 6.2.

Table 6.2 Results of β-galactosidase assays

Reporter construct	No plasmid	pBMPRP2	pBMprp2-9	pSB1(prp16-1)
pYAHB2wt	100%	100%	100%	100%
pYAHB2A1	-	-	-	-
pYAHB2C1	-	-	-	-

pYAHB2A5	45%	45%	45%	45%
pYAHB2C259	8%	19%	18%	23%

Table 6.2. Values shown as % activity of wild type intron. - indicates no detectable β-galactosidase activity (less than 1%). All cultures were grown in drop-out galactose medium, maintaining selection for the two plasmids.

6.2.5 Copper resistance assays.

The figures for the β-galactosidase assays were averaged over several experiments, as the assay can be rather variable. To confirm these results, another reporter system was obtained (Burgess and Guthrie, 1993). This time, the *CUP1* gene, which allows growth on copper, was fused to *ACT1* instead of the *lac*Z gene (Figure 6.6).

Figure 6.6. Copper reporter constructs.

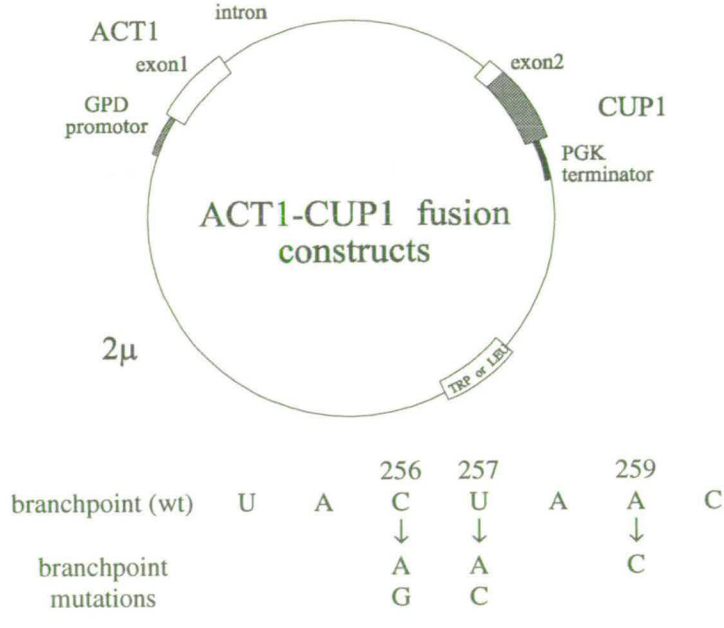


Figure 6.6. These reporter constructs were used in *CUP1*Δ strains 46 and 51, so that the level of splicing could be measured indirectly by growth on plates containing different concentrations of copper. Alleles of *PRP2* or *Prp16* to be tested were co-transformed into the *CUP1*Δ strains with the reporter constructs. Using *TRP1* as a selectable marker for the reporter constructs and *URA3* as a marker for the alleles to be tested, co-transformants were readily selected on plates lacking tryptophan and uracil.

The ability of *cup1Δ* cells to grow in the presence of increasing concentrations of copper is proportional to the level of expression of the *ACT1-CUP1* fusion, which is limited by the efficiency of splicing of the *ACT1* intron. The following results were obtained by suspending colonies in sterile distilled water and spotting the cell suspension onto plates containing different concentrations of copper, with glucose as the carbon source.

Mutations in all the conserved sequences in the intron were tested with these *PRP2* alleles, and also wild-type and mutant *prp16*. Again no suppression was seen of the 5' splice site mutations. However, there was some suppression in the case of some branchpoint mutations.

Table 6.3 Level of copper tolerance on copper/glucose plates (mM Cu^{2+}).

Reporter Plasmid	Intron Mutation	pSB1 (prp16-1)	pG1PRP 16epit	pBMPRP2	pBMprp2-9	pBM125 (vector)
pG14	wild-type	1.0	1.0	1.0	1.0	1.0
pSB47	C256A	**0.18**	**0.18**	0.10	0.10	0.10
pSB48	C256G	0.10	0.10	0.10	0.10	0.10
pSB49	U257A	0.05	0.05	0.05	0.05	0.05
pSB50	U257C	0.10	0.10	0.10	0.10	0.10
pG100	A259C	**0.25**	**0.25**	*0.18*	*0.18*	*0.18*

Strong suppression is shown in bold, with weaker suppression shown in italics. pG100 levels differ slightly from published levels (Lesser and Guthrie, 1992)

In most cases, the ability of each reporter gene to support growth in the presence of copper is similar to the published results and indicates allele-specific suppression of two branchpoint mutations by *prp16-1*. However, suppression of two branchpoint mutations upon overexpression of wild type *PRP16* was novel.

Table 6.4. Level of copper tolerance on copper/galactose plates (mM Cu^{2+}).

Reporter Plasmid	Intron Mutation	pSB1 *(prp16-1)*	pG1PRP16 epit	pBMPRP2	pBMprp2-9	pBM125 (vector)
pG14	wild type	1.0	1.0	1.0	1.0	1.0
pSB47	C256A	*0.18*	**0.25**	**0.25**	**0.25**	0.10
pSB48	C256G	0.10	0.10	0.10	0.10	0.10
pSB49	U257A	0.05	0.05	0.05	0.05	0.05
pSB50	U257C	0.10	0.10	0.10	0.10	0.10
pG100	A259C	**0.25**	**0.25**	**0.25**	**0.25**	*0.18*

Strong suppression is shown in bold, with weaker suppression shown in italics.

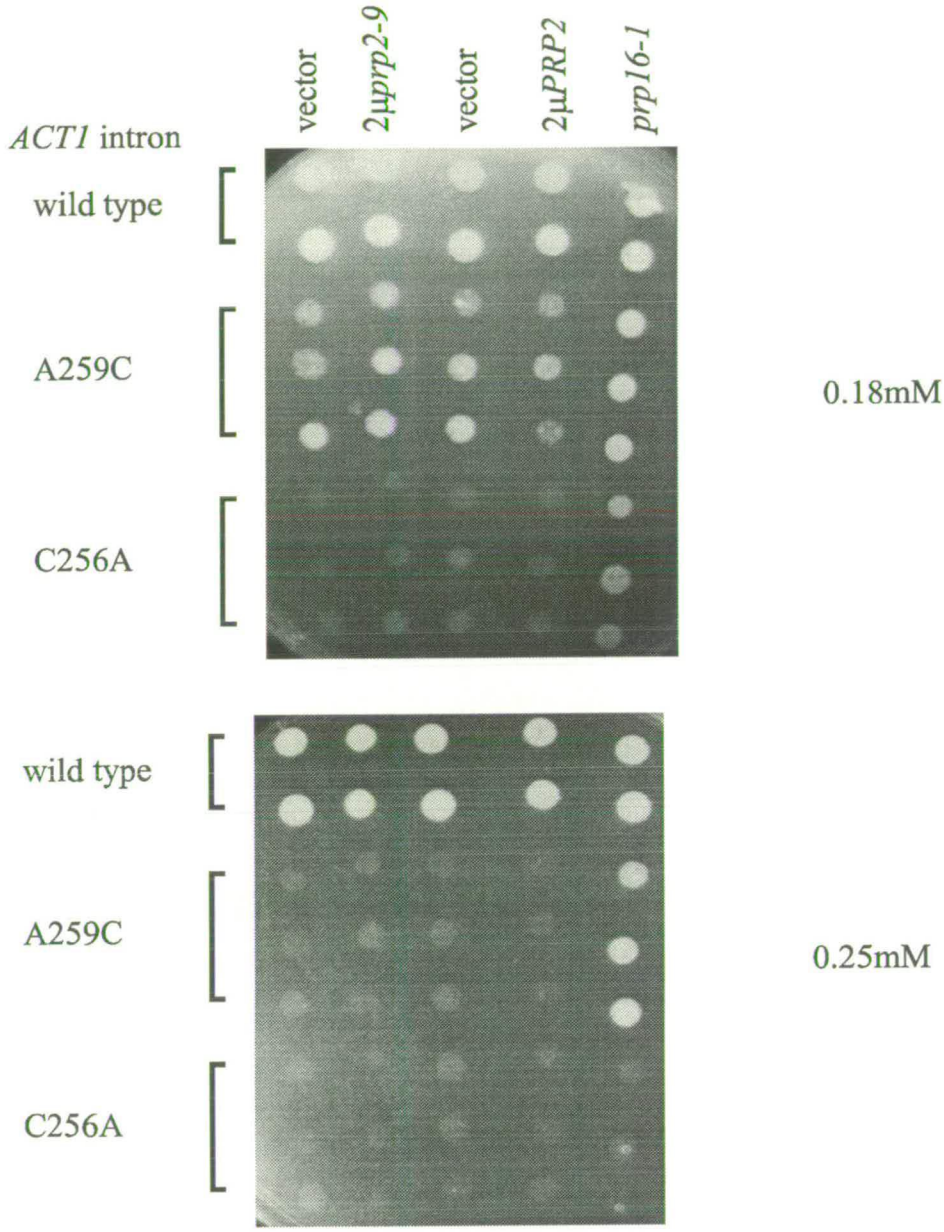
On the galactose plates, there was more growth on higher levels of copper, particularly in the case of the overexpressing *PRP2* transformants which grew in the presence of 0.25mM copper. However, the vector control was also growing at a higher level of copper.

Several temperature-sensitive splicing mutants are known to self-suppress when grown on non-glucose carbon sources. An allele of *REG1* (Tung *et al*, 1992), a negative regulator in glucose repression, has been shown to suppress several splicing mutants including *rna1-1*, *prp2-1* and *prp6-1*. In the case of *rna1-1*, suppression is caused by the mutant *reg1* allowing overproduction of rna1-1 protein. Also, *rna1-1* and other splicing mutants are known to self-suppress at high gene copy number.

It is exciting to hypothesise that the observed suppression of certain branchpoint mutations during growth on galactose may be a consequence of elevated Prp16 protein, which may be able to suppress the branchpoint mutation in the *ACT1* intron. However, these data indicate that high level expression of Prp2 protein may enhance the suppression.

To clarify the situation with respect to possible suppression by Prp2 protein, two alleles of *PRP2* were tested using high copy-number plasmids with the gene under the control of the *PRP2* promoter. These plasmids constitutively overexpress the *PRP2* alleles on glucose.

Figure 6.7 Copper resistance assays.



The results showed that cells constitutively expressing *PRP2* grew only on the same copper concentration as the vector control, with only *prp16-1* growing on high copper.

6.3 Discussion.

It has been shown that although the *prp2-9* mutation does not eliminate Prp2 function, it decreases the efficiency of the Prp2 protein in the sense that for complementation of the

prp2-1 ts growth defect to take place, the protein must be produced at higher levels than the wild-type Prp2. Unlike *prp16-1*, overexpression of prp2-9 does not cause a growth defect at low temperature.

In the study of suppression of intron mutations, an interesting carbon-source effect came to light. It was also observed that overexpressing the wild-type *PRP16* gene leads to suppression with the same allele specificity and similar efficiency to that of the *prp16-1* mutation. In addition, growth of cells on galactose results in a similar suppressor effect, possibly due to increased levels of Prp16 protein. However *prp16-1* is able to suppress the branchpoint mutations even at low copy.

Many studies have been carried out to investigate splicing complex formation on transcripts with mutant intron sequences. Early *in vitro* studies failed to show binding of snRNPs to mutant *ACT1* transcripts. Vijayraghaven (Vijayraghaven *et al*, 1986) used immobilised labelled transcripts on a column to isolate splicing complexes. Splicing extracts were loaded onto the column and splicing complexes formed on the labelled pre-mRNA were eluted after washing. Native gel electrophoresis was then used to investigate resulting protein/RNA complexes. These experiments failed to show binding of the U1 snRNP or any other snRNP to RNA containing a mutant branchpoint sequence. This was unexpected at the time as it was thought that the U1 snRNP would bind to the 5' splice site irrespective of the sequence of the branchpoint.

Cheng and Abelson (1987) and Ruby and Abelson (1988) also looked at complex formation on *ACT1* transcripts containing either C256A or A259C branchpoint mutations. Again no complex formation was seen *in vitro*.

The results of these experiments are seemingly at odds with the suppression studies of the mutant branchpoint by *PRP16*. Assuming that Prp16 functions only at step 2, and that Prp16-mediated suppression also occurs at step 2, these transcripts must be assembled into spliceosomes and proceed through step 1. However, the network of interactions which allow this to occur *in vivo* may somehow be inhibited or disrupted *in vitro*.

More recent studies in mammalian *in vitro* systems have shown that complexes do form on transcripts containing mutant branchpoints (Query *et al*, 1996). Mammalian splicing requires a less highly conserved branchpoint sequence than yeast, but the branch adenosine residue is invariant. Nevertheless, all of the transcripts containing a modified branch residue formed splicing complexes. Interestingly, it was found that formation of complexes was strongly stimulated by the 5' splice site and only minimally affected by branch site mutations.

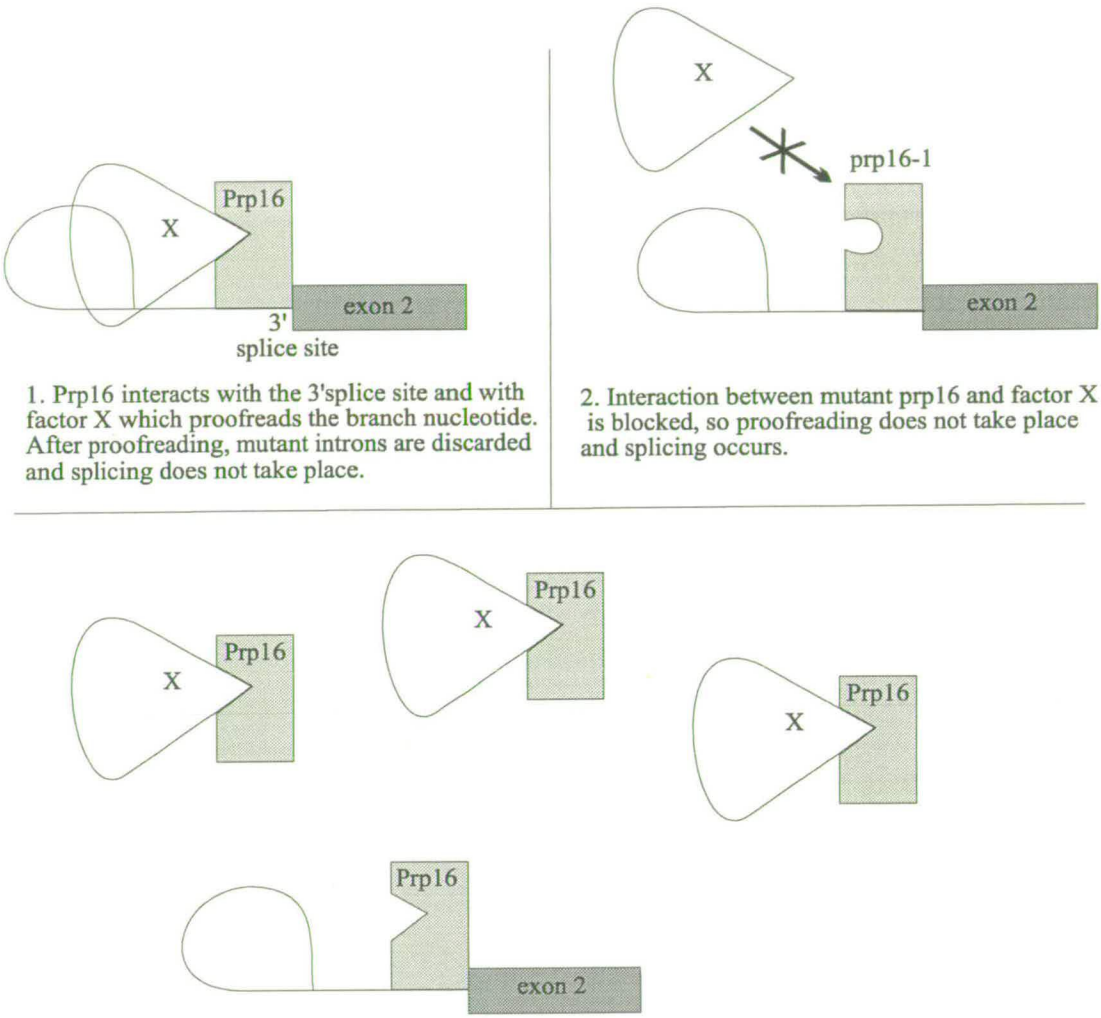
If the same is true in the case of yeast introns i.e. the branchpoint proofreading step occurs after step 1, this is compatible with the observed lack of suppression by Prp2. Prp2 is known to act at step 1 (King and Beggs, 1990; Lin) and Query's results suggest that mutant introns are assembled into spliceosomes and step 1 is carried out. This leaves a lariat-exon 2 intermediate with an abnormal branch which may be suppressed by *prp16* mutants.

It was found that the same suppressor effect displayed by the *prp16* mutants (Burgess and Guthrie, 1993) can be seen simply by overexpressing wild-type *PRP16*.

Prp16 protein is known to bind to the spliceosome after lariat intron-exon 2 formation (Schwer and Guthrie, 1991). It can be specifically crosslinked to the 3' splice site of the intron (Umen and Guthrie, 1995) and has been shown to induce a conformational change in the spliceosome (Schwer and Guthrie, 1992).

This would suggest that perhaps Prp16 is not directly involved in proofreading the branchpoint, but may interact with a factor which does. All 6 mutant suppressor alleles of *prp16* isolated by Burgess and Guthrie were found to have reduced ATP hydrolysis activity. This has been proposed to be the basis of suppressor activity (Burgess and Guthrie 1993), however, this would not account for the suppression seen by overexpression of wild-type *PRP16*. The following model proposes a mechanism whereby Prp16 might mediate or modify the binding of a proofreading factor, X. When X is unbound or bound in an inactive conformation, splicing occurs. When X binds to an aberrant branchpoint, a discard pathway is activated and splicing does not occur. The model suggests ways by which X may be unable to activate the discard pathway in the presence of either mutant prp16 or overproduced wild-type Prp16 proteins.

Figure 6.8 Model suggesting mechanism of suppression by single-copy *prp16* alleles and by overexpression of wild-type *PRP16*.



This differs from the published model. Burgess and Guthrie propose that as all the suppressor mutants have lowered ATPase activity relative to that of wild-type Prp16, the suppressor phenotype might arise as a consequence of alterations in the rate of ATP hydrolysis. This is likened to the kinetic proofreading models proposed for translation and DNA replication (reviewed by Burgess and Guthrie, 1993). However, in these two cases, fidelity is lowered when the rate of NTP hydrolysis is raised. These proofreading systems, protein synthesis and DNA replication are both polymerisation processes, and splicing is not.

In the case of the above model, interaction with factor X is necessary for proofreading but not for step two of splicing to occur. The rate of ATP hydrolysis by Prp16 protein may not directly influence the proofreading step.

Protein factors which interact with Prp16 could be sought by using the two-hybrid system (Fields and Song, 1989) to screen a library with Prp16 as bait. Any interacting factors found could be overexpressed in the presence of the Prp16 branchpoint suppressor mutants to test for restoration of the fidelity of splicing.

Chapter seven.

Summary and future work.

Since *PRP2* was first cloned and sequenced (Lee et al, 1984: Chen and Lin, 1990) much has been learned about the role in splicing of the Prp2 protein. In order to further dissect the function or functions of the Prp2 protein in the splicing process, several regions were targeted for mutagenesis.

The first region of interest was the putative zinc finger motif which spans the *Pvu*II site towards the 3' end of *PRP2*. Conservative substitutions appeared to have no detectable effect on the function of Prp2 as determined by complementation assays. Only more major changes, namely the non-conservative cys2 to tyr substitution and the 4-amino acid insertion had any significant effect in complementation assays when overexpressed in a *prp2-1* background. This was in contrast to their effect in a background disrupted for *PRP2* when, on expression form the *PRP2* promoter, no growth defect was detected. The value of these mutations lies in the possibility that the Prp2 protein has more than one domain capable of interacting with RNA. Future mutational analysis of putative RNA-binding sites may use the zinc finger mutations in a search for synthetic lethals. Mutations in two distinct domains which appear to have no effect on Prp2 function alone may prove to be detrimental when in combination.

Chapter four discusses the effect of deleting domains from Prp2 proteins carrying dominant-negative mutations. In the case of one dominant negative mutation, $PRP2^{AKT}$, removing either of the two domains downstream of the dominant mutation did not abrogate poly(U) binding by the protein, although the bound protein was eluted from poly(U)-agarose at a slightly lower salt concentration than wild-type protein. A continuation of this deletion analysis may result in elucidation of RNA binding sites in Prp2. There is also a possibility that Prp2 interacts with one or more other protein factors during the course of the splicing reaction. The use of truncated proteins which retain some activity of Prp2 can be used to investigate which domains of the protein are necessary for protein/protein interactions.

In an attempt to determine if the Prp2 protein had an activity involved in the transition of splicing complex III to complex I, a system was developed for the *in vivo* and *in vitro* depletion of Prp2. However, inherent problems prevented this strategy from becoming viable. The possibility that either the *in vivo* or the *in vitro* depletion of Prp2 results in the depletion of one or more additional splicing factors is a major obstacle to this approach.

Finally, a series of experiments to determine a possible proofreading role for Prp2 in splicing were carried out. The results detailed in chapter six indicate that Prp2 has no role in proofreading either the 5' splice site or the branchpoint sequence. Intriguingly, it was discovered during the course of experiments with Prp2 that the overproduction of wild type Prp16 protein had a similar effect to that previously published for the prp16-1 mutant. This has led to a model proposing how Prp16 is involved in the proofreading of the branchpoint sequence and how suppression by both prp16-1 and wild type Prp16 protein is possible.

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