A FUNCTIONAL ANALYSIS OF Prp45p, A PRE-mRNA SPlicing FACTOR IN
Saccharomyces cerevisiae

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DECLARATION

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

I would like to thank you, Jean; your trust in me, patience and encouragements meant a lot to me. I am aware that I wouldn’t have done this piece of work without my colleagues, past and present members of Beggs lab, and especially without Joanna; many thanks for your help, guys, and for the good working atmosphere and nice moments we’ve shared.

I am very grateful to Professor Ken Murray and to the Darwin Trust of Edinburgh who made possible this dream become true...

Many thanks to all my friends from home and all over the world: to Anca, Irina, Cristina and Paul for always reminding me that there is only one pair of steps in the sand...and to my friends here, in Edinburgh, especially to Adriana and Nicu for taking good care of me in the last days.

To my co-pilot, George...with the words we’ve been waiting for long: we did it! and with the promise...

And I would especially like to thank my parents for so many years of understanding, patience and support and love.
I dedicate this thesis to my dearest sister, Adelina, for her faith has kept the light lit throughout these years.
ABSTRACT

Nuclear pre-mRNA splicing in eukaryotes involves the removal of the intervening sequences (introns) from pre-messenger RNA by a two-step trans-esterification mechanism. This process occurs on a large ribonucleoprotein particle called the spliceosome, which consists of five small nuclear RNAs (snRNAs) and a number of protein factors. The spliceosome is a highly dynamic machine and over fifty known protein factors mediate its assembly and function.

One of these protein factors in the yeast *Saccharomyces cerevisiae* is Prp45p (the name derived from its involvement in pre-mRNA processing). The 42kDa Prp45p is essential for pre-mRNA splicing. It was previously shown that Prp45p is associated with an Ntc-protein complex and directly interacts in vivo with the splicing factor Prp46p, another member of this protein complex. Apart from this, in recent years, extensive research was done for the homologues of Prp45p in human and *Drosophila melanogaster* regarding the role of these proteins as regulators of transcription in different pathways.

In this study we aimed to understand the role Prp45p executes within the spliceosome and the splicing mechanism. Part of this work was a detailed investigation into the relationships between the structure and the function of this protein by employing a screen for temperature-sensitive mutants.

Prp45p is known to associate with the spliceosome throughout the splicing reactions but at what stage the protein is involved in spliceosome assembly i.e. pre-spliceosome, inactive or active spliceosome remained to be determined. Using a *PRP45* conditionally regulated strain it was found, upon depletion of Prp45p from yeast cells and analysis of the in vitro splicing systems by native gel fractionation, that the formation of the active spliceosome does not take place. This behaviour is compatible with Prp45p being a component of the Ntc-protein complex, which is known to be involved in this step of spliceosome assembly. Moreover, co-
immunoprecipitation experiments with a tagged allele of the splicing factor Prp46p, confirmed the interaction between these two proteins suggested by two-hybrid screens.

Using random PCR mutagenesis there were identified two mutants with growth defects at 37°C. The mutants, named \textit{prp45-57} and \textit{prp45-113}, contained mutations in two regions, designated \textit{A} and \textit{B} and located respectively upstream and, downstream of the absolutely conserved SNWKN motif. To understand which of the mutations were responsible for the temperature-sensitive phenotype, the substitutions in region \textit{A} and/or region \textit{B} were recreated by site-directed mutagenesis. It was proved that the phenotype requires mutations in both segments, which strongly suggests that regions \textit{A} and \textit{B} together play roles in protein function, perhaps through intra- or inter-protein interactions. However, co-immunoprecipitation experiments revealed that these substitutions in Prp45p do not alter the interaction with Prp46p \textit{in vitro}. In order to investigate the role of these mutations within the cell, two strains were created that carry these mutations in a c-Myc tagged \textit{PRP45} ORF. When growing these strains at non-permissive temperature and employing a β-galactosidase assay, it was found that they had a mild effect on pre-mRNA splicing and do not affect the transcription/translation of the reporter genes. These results were confirmed by microarray analysis and by Northern blotting showing that introns are not accumulating but the intron-encoded snoRNA species do. This indicates that Prp45p could be involved in other metabolic processes like pre-mRNA turnover.
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<tr>
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<td>BP</td>
<td>Branch point</td>
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<td>Ribonuclease</td>
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<td>Ribonucleoprotein</td>
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</tr>
<tr>
<td>RT</td>
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<tr>
<td>rpm</td>
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<td>SDS</td>
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<td>$N,N,N',N'$-tetramethyl-ethylenediamine</td>
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<td>tRNA</td>
<td>transfer Ribonucleotide Acid</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Ts</td>
<td>Temperature-sensitive</td>
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<td>β-Gal</td>
<td>β-Galactosidase</td>
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<tr>
<td>μF</td>
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<td>μg</td>
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<td>Microgram(s)</td>
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<td>UV</td>
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<tr>
<td>V</td>
<td>Volt(s)</td>
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<tr>
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<td>Volume per unit volume</td>
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<tr>
<td>W</td>
<td>Watt(s)</td>
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<td>w/o</td>
<td>Without</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per unit volume</td>
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<td>YMGRS</td>
<td>Yeast Medium Galactose/Rafinose/Sucrose</td>
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<tr>
<td>YMM</td>
<td>Yeast Minimal Medium</td>
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<td>YPDA</td>
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<td>YPGalA</td>
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1.1 Pre-mRNA splicing

In 1977 it was discovered that most of the nuclear protein-coding genes are discontinuous, being interrupted by non-coding intervening sequences known as introns (Berget et al., 1977; Chow et al., 1977; Abelson, 1979). All the information within the genes, including introns, is transcribed into pre-mRNA. Then, the introns are excised within the nucleus and the coding sequences or exons are joined to form the mature mRNA that is subsequently exported to the cytoplasm. This process is called pre-mRNA splicing and since then intensive research has been performed to understand its molecular mechanism.

The use of either nuclear extract of HeLa cells or whole extract of yeast cells has facilitated the understanding of the catalytic mechanism of splicing and the biochemistry of the cellular components of this process. However, although the basics are the same, there are a lot of differences among eukaryotes from the number and size of introns and exons to the function and organisation of the splicing machinery. For instance, in higher eukaryotes, a gene can contain more than 50 introns and the introns can have up to 200,000 nucleotides in length. In contrast, in the unicellular eukaryote *Saccharomyces cerevisiae* (referred to here as a yeast) only 4% of the 6000 genes have introns (most of them having only one) with an average size of 600 nucleotides.

There are some important traits of splicing conserved among yeast and higher eukaryotes (Ruby et al., 1991). Firstly, the molecular organisation of the introns. These are: three short conserved sequences required for splicing: the 5'splice site (5'SS), the 3'splice site (3'SS) and the so-called branchpoint sequence (BP) (Figure 1.1). In yeast these sequences are strongly conserved while in mammalian cells they are less conserved. The also contain a pyrimidine-rich region (Py)$_n$ upstream of 3'splice site.
**Saccharomyces cerevisiae**

![Diagram of conserved sequences in *S. cerevisiae*](image)

**Mammals**

![Diagram of conserved sequences in mammals](image)

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**Figure 1.1 Schematic representation of the conserved sequences within the introns in *S. cerevisiae* and mammals.**
The three sequence elements: the 5' splice site, the branchpoint and the 3' splice site are indicated. \((Y)_n\) represents the polypyrimidine tract, Y-pyrimidine, R-purine, N-any base.

A second common feature between all eukaryotes is the chemistry of the splicing reaction: an ATP-dependent process consisting in two successive trans-esterification reactions (Figure 1.2). In the first step the 2'OH group of a specific adenosine, the branchpoint nucleotide, attacks the phosphate at the 5'splice site. In the second step the 3'OH group of the 5'exon attacks the phosphate at the 3' splice site resulting in the ligation of the two exons. The intron is liberated and degraded.
Figure 1.2 Catalytic steps of nuclear pre-mRNA splicing. Exons are shown as boxes and the intron as a line. The dashed arrows symbolise the nucleophytic attack of the hydroxyl groups at the splice site.
1.2 The spliceosome

1.2.1 Spliceosome Components

Nuclear pre-mRNA splicing requires the formation of a large, dynamic ribonucleoprotein complex, the spliceosome. This is composed of small nuclear ribonucleoprotein (snRNP) particles and non-snRNP protein factors. Five small nuclear RNA molecules (U1, U2, U4, U5 and U6 snRNA) associate with numerous proteins to form spliceosomal snRNPs. Regarding these snRNA-associated proteins, they can be classified into two groups. One consists of core or Sm proteins (B, D1, D2, D3, E, F and G as they were originally named in metazoa) shared by U1, U2, U4 and U5 snRNPs but not by the U6 snRNP; b) proteins specific for a certain snRNP (Will et al., 1997).

The pathways by which snRNPs are formed are evolutionarily highly conserved in eukaryotes although there are differences between U6 snRNP biogenesis and the other four snRNPs (for review, Will and Luhrmann, 2001). First of all, U1, U2, U4, and U5 snRNAs are transcribed by RNA polymerase II and are 5’end capped (7-methyl guanosine cap structure) and then exported to the cytoplasm where they associate with the core proteins. The Sm/core proteins form a ring-like structure that binds through a specific amino acid motif (Sm motif) to the conserved Sm-site present in all these snRNAs (Lazar et al., 1982). This association promotes hypermethylation of the snRNA cap structure and 3’end processing. In addition to this, the U snRNAs are internally modified before their association with proteins. These chemical modifications consist of 2’-O-methylation of ribose moieties and pseudouridylation (Yu et al., 1998) and, at least for U5 snRNA, they are generated by small nucleolar RNP (snoRNPs) particles (Jady and Kiss, 2001). SnoRNPs are nucleolar particles composed of snoRNAs and associated proteins and some are known to be needed for processing of ribosomal RNA, like cleavage, 2’-O-methylation and pseudouridyllation. After the addition of the snRNP-specific proteins the newly assembled snRNP is imported into the nucleus where it contributes to the
spliceosome assembly and/or splicing reaction.

As for U6 snRNP, its snRNA is transcribed by RNA polymerase III, acquires a different 5' cap (γ - monomethyl cap structure) and does not present the Sm-binding site. As a consequence, U6 snRNA undergoes processing pathways different from the other spliceosomal snRNA, i.e. it is not exported into the cytosol and hypermethylated / associated with Sm proteins. However, sequence similarities and biochemical analyses (Fromont-Racine et al., 1997) led to identification of nine proteins that contain the Sm motif. Therefore they were called Sm-like proteins or Lsm proteins. Seven of these assemble ring like-structures (Achsel et al., 2001) that perform different functions within the cell (Mayes et al., 1999). Thus, Lsm2-8p is involved in pre-mRNA splicing, U6 accumulation, U6 snRNP biogenesis, and in processing and stability of stable RNAs, including rRNA (Mayes et al., 1999; Salgado-Garrido et al., 1999; Kufel et al., 2003) while Lsm1-7p complex functions in cytoplasmic mRNA degradation promoted by mRNA decapping (Boeck et al., 1998; Tharun et al., 2000). U6 snRNA is also pseudouridylated and 2'-O-methylated, processes catalysed by snoRNPs (Ganot et al., 1999).

1.2.2 Spliceosome Assembly

Spliceosome assembly is an ordered, dynamic process, which involves multiple RNA-RNA, RNA-protein and protein-protein interactions. The earliest step in spliceosome assembly is the binding of U1 snRNP to the 5'splice site of the pre-mRNA in an ATP-independent manner (Figure 1.3). The complex formed – commitment complex (CC) or early complex (E) – is very stable due to the base-pairing interactions between complementary sequences of U1 snRNA and the conserved 5' splice site. This commitment complex triggers the addition of U2 snRNP and its binding via Watson-Crick base pairing with the branchpoint sequence. The new complex is called pre-spliceosome/complex A/complex III. The branchpoint adenosine (UACUAAC) is excluded from this base-pairing and thus it is activated for the specific attack of the 5'splice site.
Figure 1.3 Schematic spliceosome assembly pathway.
U1 snRNP binds to the 5' splice site (5' SS) to form the commitment complex (CC). U2 snRNP binds to the branchpoint (BP) to form the pre-spliceosome. Pre-formed U4/U6,U5 tri-snRNP associates to form the inactive splicing complex. This loses the U1 and U4 snRNPs and turned into an active spliceosome able to perform the two steps of splicing. The trans-esterification reactions finished, mature mRNA is released and spliceosome components are dissembled and then recycled. The intron lariat product is linearized by debranching enzyme and degraded.
Subsequently, the pre-formed U4/U6.U5 tri-snRNP and other non-snRNP factors are incorporated to create a complex that is unable to support splicing and thus denominated inactive spliceosome/complex B/complex II. The spliceosome now undergoes a conformational rearrangement which involves the formation of new and/or rearrangement of existing protein-RNA and RNA-RNA interactions. For instance, U4/U6 and U1 snRNA-5'splice site basepairing are destabilised and RNA-RNA interactions between U2 snRNA and U6 snRNA promote the formation of the catalytic centre of splicing (Madhani and Guthrie, 1992; Field and Friesen, 1996). This step of the cycle produces the active spliceosome (or complex C/II), which performs the two trans-esterification reactions. The U5 snRNA also plays a role in both steps of splicing reaction. The highly conserved loop in the U5 snRNA molecule interacts with exon sequence at the 5' and the 3' splice sites. In this way the U5 loop probably aided by U5 snRNP proteins such as Prp8p can anchor and tether the 5'exon splicing intermediate produced by the first transesterification, and then assist the alignment of the 5' and the 3' exons for the second catalytic step (Newman and Norman, 1992; Sontheimer and Steitz, 1993; Newman, 1997; Dix et al., 1998).

The two reactions completed, the spliced mRNA is released, the spliceosome disassembles and the snRNPs and non-snRNP proteins can reassemble onto other pre-mRNA molecules.

However, recent studies promote another theory, namely the existence of a 45S penta-snRNP complex comprising all five snRNAs and the associated proteins, which binds the pre-mRNA substrate as a whole rather than discrete particles as previously described (Stevens and Abelson, 2002). This complex was purified from yeast extract at low salt concentration (50 mM) compatible with the in vitro splicing conditions in contrast with the previous studies in which snRNP particles have been analysed under higher salt concentrations (150 – 250 mM) that could destabilise the complex. This model suggests that pre-mRNA interacts with the pre-formed penta-snRNP, the subsequent steps being the traditional ones: the 5' splice site base pairing to the U1 snRNA and the branchpoint binds to the U2 snRNA, followed by the displacement of U1 snRNA by 5' splice-site - U6 snRNA interaction. Alternatively, a discrete U1 snRNP initially interacts with pre-mRNA substrate and the preformed
tetra-snRNP complex is recruited and the assembly and subsequent splicing reactions proceed as usual.

Concurrent with spliceosome assembly, activation, chemical modification of the pre-mRNA substrate, then mRNA release, there are important alterations in the constituents and conformation of the spliceosome. It is believed that ATP hydrolysis drives these rearrangements of RNA-RNA and RNA-protein interactions (for review, see Staley and Guthrie, 1998; Schwer, 2001). Some proteins required for pre-mRNA splicing possess a set of sequence motifs conserved among ATPases. The proteins proposed to play such a role in splicing belong to so-called the DEAD/H-box protein family. This family of proteins is defined by the presence of eight conserved domains, one of which is the DEAD/H motif responsible for ATPase function while another, the SAT domain, plays a role in RNA unwinding. This is the reason why these proteins are thought to be RNA-dependent ATPases and ATP-dependent RNA helicases, although the latter role has been experimentally proved only for some members of the family. Each of these factors is involved in a different stage of the splicing cycle (Lin et al., 1987; Burgess et al., 1990; Chen and Lin, 1990; Company et al., 1991; Schwer and Guthrie, 1991; Arenas and Abelson, 1997).

Thus Prp28p is involved in unwinding the U1-5’splice site sequence (Chen et al., 2001) while Brr2p is believed to unwind the U4/U6 duplex (Raghunathan et al., 1998), both events are leading towards the activation of the spliceosome.

ATP hydrolysis by Prp2p is required for activation of the spliceosome prior to the step 1 trans-esterification (Kim et al., 1996). This hydrolysis is followed by a change in the pre-mRNA structure promoting the first splicing reaction. The specificity of its action might be controlled by Spp2p which can act as a mediator between Prp2p and the spliceosome (Roy et al., 1995).

The 121 kDa Prp16p is essential for step 2 (Schwer and Guthrie, 1991). Importantly, purified Prp16p has been shown to exhibit ATP-dependent RNA helicase activity in vitro (Wang et al., 1998). ATP hydrolysis by Prp16p elicits a conformational change in the spliceosome that results in protection of the 3’splice site from RNase H prior to the second transesterification reaction (Schwer and Guthrie, 1992). To induce the conformational changes, Prp16p needs the recruitment
of splicing factors Slu7p, Prp18p and Ssf1 to the spliceosome (Ansari and Schwer, 1995; Brys and Schwer, 1996). Slu7p and Prp18p are required in 3' splice site selection especially when the distance between the branchpoint and the 3'splice site is longer than 12 nucleotides (Brys and Schwer, 1996; Zhang and Schwer, 1997).

Prp43p was originally discovered as a DEAH-box protein sharing sequence homology with the Prp2p, Prpl6p and Prp22p. It was shown that Prp43p is involved in disassembly of the spliceosome after the release of mature mRNA (Arenas and Abelson, 1997; Company et al., 1991).

Prp22p (130 kDa) was first isolated in a screen for temperature-sensitive mutants in pre-mRNA splicing (Vijayraghavan et al., 1989). Two different roles have been proposed for the functions of Prp22 protein. Firstly, Prp22p is required for the release of mRNA from the spliceosome after the second step of splicing. Experiments using nuclear extracts from a *prp22-1* temperature-sensitive strain showed the retention of the mRNA and intron-lariat products of splicing within the spliceosome parallel with the accumulation of unspliced pre-mRNA (Schwer and Gross, 1998; Company et al., 1991). It is thought that ATP hydrolysis by Prp22p determines rearrangements within the spliceosome and drives the release of mRNA from the complex by disrupting RNA-RNA interactions (Schwer and Gross, 1998). Secondly, Prp22p is required during the second step of splicing in an ATP-independent manner. The involvement of the protein is necessary only when the distance between the branchpoint and the 3' splice site is longer than 21 nucleotides (Schwer and Gross, 1998). It has been suggested that Prp22p acts in concert with Slu7p and Prp18p to protect the 3' splice site from the activity of RNase H and further to position for catalysis the 3' splice site PyAG and the 3'OH terminus of the 5'exon (Schwer and Gross, 1998). Using crosslinking analysis it was determined that Prp22p transiently contacted the 3' splice site between the first and the second step of splicing and this association is dependent on Prp16p (McPheeters et al., 2000).

Following the discovery of the splicing process, many experimental lines were followed to identify the components of spliceosomal machinery and their functions. In yeast the use of genetic analyses like random screens for temperature/cold-sensitive mutants, two-hybrid screens, suppressor screens or synthetic lethal screens
led to identification of many splicing factors (for review, see Beggs, 1995 and references therein). Sophisticated proteomic analyses of yeast and mammalian cell extracts have been developed in recent years. They involve affinity purification of protein/spliceosome/snRNP complexes using well-characterized and tagged splicing factors or pre-mRNA substrates, followed by mass spectrometry to identify the protein components (Ohi et al., 2002; Jurica et al., 2002; Makarov et al., 2002; Stevens and Abelson, 2002).

One of the splicing factors identified by two-hybrid screens and then by biochemical analyses is Prp45p and this is the subject of the present work.

1.3 Prp45 Protein

1.3.1 Prp45p Structure

The \textit{PRP45} gene was first identified in a screen for essential genes on chromosome I in \textit{S. cerevisiae} and named Fun20 (Fun = function unknown) (Diehl and Pringle, 1991). \textit{PRP45} is essential for cell viability, as shown by Diehl and Pringle, 1991 and confirmed by Albers et al., 2003.

The 379 amino acid Prp45p has a molecular weight of 42.4 kDa and a hydrophilic character. Due to its amino acid sequence within the N-terminal/central region, Prp45p was assigned to be a member of the SNW protein family found in many eukaryotes from the simple parasite \textit{Encephalitozoon cuniculi} to human. The name of this family derived from a sequence, SNWKN, which is highly conserved among the members with the exception of \textit{E. cuniculii} in which the asparagine residue (N) substituted with methionine (M) (Figure 1.4). The conserved nature of this motif does not confer specific function to the protein, at least in yeast, as Martinkova et al. (2002) showed that the substitution of the SNW residues (170SNW172) with triple alanine was compatible with cell viability.
Figure 1.4 The alignment of *S. cerevisiae* NH₂-terminal/central domain with the putative members of SNW family.

The sequences were identified using NCBI BLAST and then aligned using Clustal W (1.82). The identities and similarities are emphasized using BOXSHADE 3.21. The conserved amino-acids are in bold and colored in red; the semi-conserved ones are in blue. The conserved SNWKN is highlighted in black.

The blue bars above the alignment delineate the regions A and B where specific amino acid residues (highlighted) are mutagenized in this work (Chapter 4).
Figure 1.4 (continued)

The gene names and accession numbers are: P. falciparum – Plasmodium falciparum (GI:38445299); P. yoelii – Plasmodium yoelii (gi:23479956); A. thaliana – Arabidopsis thaliana (GI:3540201); H. vulgare – Hordeum vulgare (GI:27948454); D. melanogaster – Drosophila melanogaster (BX42); A. gambiae – Anopheles gambiae (gi:1209709); H. sapiens – Homo sapiens (GI:19172986); M. musculus – Mus musculus (GI:11595618); S. japonicum – Schistosoma japonicum (gi:29841015); C. elegans – Caenorhabditis elegans (Q22836); N. crassa – Neurospora crassa (GI:11596618); S. pombe – Schizosaccharomyces pombe (GI:4678690); D. discoideum – Dictyostelium discoideum (SNWA); S. cerevisiae – Saccharomyces cerevisiae (Prp45p); E. cuniculi – Encephalitozoon cuniculi (gi:19172986).
Baudino et al. (1998) defined three important domains in human homologue of Prp45p, NcoA-62/SKIP, that are fairly conserved (Figure 1.5). These regions are: a) the SNW domain that in yeast Prp45p is located between amino acid residues 120 and 230; b) a proline-rich domain upstream of SNWKN motif that is shared by all organisms except S. cerevisiae. It is considered that this sequence constitutes a potential binding site for SH3 domains (src homology domain 3) (for review, Zarrinpar et al., 2003) and this facilitates interactions with other proteins; c) the C-terminal region of SNW proteins contains an α-helix and three β-strands that resemble the consensus sequence of SH2 domains (src homology domain 2) (Figure 1.5). SH2 domains are mainly found in tyrosine kinases involved in signal transduction cascades and they recognise the phosphotyrosines on the surface of proteins. In yeast Prp45p the SH2 domain is poorly conserved and rather degenerated and the whole C-terminal region was shown to be dispensable for cell survival (Martinkova et al., 2002). Yeast Prp45p also lacks two C-terminally located highly charged regions common for its homologues (Figure 1.5) that seem to play crucial roles within transactivation processes, aspects discussed in the following sections.

So far, two different experimental lines performed on Prp45p have shown the importance N-terminal half of the protein. Firstly, complementation assays with truncated versions of the yeast Prp45p identified the minimum domain necessary for cell viability within the N-terminal 190 amino acid residues (Martinkova et al., 2002). Secondly, two-hybrid screen analyses mapped the Prp45p minimum region of interaction with Prp46p between amino acids 54 and 204 (Albers et al., 2003). An alignment of the N-/central region of the SNW proteins is depicted in Figure 1.4.

The N-terminal region of Prp45p is shorter and less conserved than its orthologs that contain conserved stretches of amino acids involved in specific functions and interactions (Figure 1.5).
Figure 1.5 Schematic diagram of the proposed homology among SNW family members.
The proteins are represented as bars. The coloured and dashed boxes indicate the common regions. Gene names and accession numbers are indicated on left. I and II are the highly charged sequences. B, C and D denominate the three β-strands.
Thus N-terminal domains of *S. pombe* Snwl (1-180) and *D. discoideum* SnwA (1-192) were found to interact with members of cyclophilin family Cyp2 and CypE, respectively (Skruzny et al., 2001). Cyclophilins are enzymes with peptidyl–prolyl cis-trans isomerase activity (PPIase) that facilitate conformational modifications of their substrates in many cellular processes like transcription, cell cycle and splicing (for review, see Hunter, 1998; Teigelkamp et al., 1998). Although these domains of Snwl and SnwA contain around 8 well-conserved prolyl-peptide bonds (Figure 1.4), subsequent analyses showed that they are not substrates of the interacting cyclophilins.

1.3.2 Prp45p Functions

1.3.2.1 Role in Splicing

Prp45p was identified as a main interactor in a two-hybrid screen with Prp22p as a bait (Albers et al., 2003). Considering this two-hybrid association with a splicing factor, it was reasonable to investigate the role in splicing of the newly identified protein. Indeed yeast cells depleted of Prp45p accumulate pre-mRNA, indicating a splicing defect *in vivo*. *In vitro* splicing assays with Prp45p-depleted splicing extract and radiolabelled substrate showed that the formation of splicing intermediates and products did not take place, indicating an *in vitro* splicing defect. Moreover the defect was partially circumvented by the addition of the recombinant Prp45p (M. Albers, PhD Thesis).

Co-immunoprecipitation of *in vitro* assembled splicing reaction with protein A-tagged Prp45p (protA:Prp45p) aimed to determine at which specific step Prp45p associates (Albers et al., 2003). The results showed that Prp45p becomes associated with the spliceosome prior to the first *trans*-esterification step and remains associated throughout the splicing process till the end of the second step when it remains associated with the excised lariat intron.

Under non-splicing conditions Prp45p associates with U2, U5 and U6 snRNAs but not with U1 and U4 snRNA, suggesting that the protein is binding to the
spliceosome after the U1 snRNP and U4 snRNP have dissociated and before the active spliceosome performs the first trans-esterification reaction (Albers et al., 2003).

As for the Prp45p homologue in human, SKIP, although there have been reports of its association with the spliceosome, a direct role in pre-mRNA splicing has not been shown till recently. Thus, in 1998 Neubauer et al. purified assembled spliceosomes by gel filtration and affinity chromatography, followed by two-dimensional gel electrophoresis to separate the components and mass spectrometry to identify them; there were identified 3 spots corresponding to SKIP. Makarov et al. (2003) using anti-SKIP antibodies precipitated spliceosome complexes and, similarly to Prp45p, determined the association of SKIP with U2, U5 and U6 snRNAs and not with U1 and U4 snRNAs. The same study showed that anti-SKIP immunoaffinity-purified both activated spliceosome and the post-splicing 35S snRNP. Thus as for Prp45p, SKIP associates with the spliceosome before the first step and dissociates following the second step together with the spliced intron. Finally, Jurica et al. (2002) affinity-purified the C complex containing splicing intermediates, followed by tandem mass spectrometry to identify the protein components. The results confirmed the presence of SKIP within the spliceosome during splicing reactions the same as for yeast Prp45p.

Relative recently a study by Zhang et al. (2003) provided functional evidence that SKIP is needed for in vivo splicing of a specific pre-mRNA (of a VDR-activated gene). A dominant negative version of SKIP (a truncated form containing amino acid residues 87-342), transfected in COS-7 cells caused the accumulation of unspliced transcripts, indicating an in vivo splicing defect.

Biochemical purification of interchromatin granule clusters (IGC) from liver nuclei followed by mass spectrometry showed that SKIP co-localizes with other splicing factors like U2AF\(^{35}\), U2AF\(^{65}\) (Mintz et al., 1999). IGCs are known to be the “headquarters” of pre-mRNA splicing factors. This result is confirmed by the interaction between S. pombe homologue, spSNW1 and spU2AF\(^{23}\) which is the small subunit of the polypyrimidine-tract binding protein spU2AF. Moreover, two-hybrid assays showed that spU2AF\(^{23}\) interacts with SNW1 homologues in Drosophila.
(Bx42), *D. discoideum* (SnwA) and *S. cerevisiae* (Prp45p). The interaction with Prp45p is very interesting knowing that *S. cerevisiae* lacks the SR proteins, including U2AF.

A Prp45p two-hybrid screen led to the identification of a novel splicing factor, Prp46p, and revealed a physical interaction between Prp45p and two other splicing factors, Syf1p and Syf3p (Albers et al., 2003). Prp46p was used as bait in a two-hybrid screen and out of all positive clones, Prp45p was statistically the most significant, followed by Syf3p.

The 451 amino acid protein Prp46p was shown to be an essential gene for cell viability (Albers et al., 2003). The Prp46p depleted cells displayed a recognisable splicing defect, the accumulation of unspliced precursor, indicating that Prp46p is also required for pre-mRNA splicing in vivo (Albers et al., 2003). Prp46p contains a cluster of seven highly conserved WD repeats (repeating units of tryptophan, W, and aspartatic acid, D) located in the central/C-terminal part. As for its function, it was suggested that the WD-repeat propeller structures create a stable platform that can reversibly form complexes with several proteins. Two-hybrid assays mapped the minimum regions of interactions within the amino acid sequence of the two proteins. Thus, Prp45p, through a portion of the conserved SNW domain (between amino acids 54 and 204) interacts with region 127–432 of Prp46p which contains all the seven WD repeats.

PRLGI, the human putative homologue of Prp46p and member of the WD family has been shown to be involved in pre-mRNA splicing (Ajuh et al., 2001). It co-precipitates the spliceosome from HeLa nuclear extracts (Neubauer et al., 1998). An interesting feature was shown for another member of the WD family in *S. pombe*, Prp5p, considered to be the homologue of Prp46p. It was determined that a temperature-sensitive *prp5-I* mutation not only causes a pre-mRNA splicing defect at the restrictive temperature (Potashkin et al., 1998) but also arrests the cell cycle in G2/M phase.

The other significant interactor either with Prp45p or with Prp46p is the splicing factor Syf3p/Clflp. It is required for both catalytic steps of splicing but the exact timing the Syf3p association with spliceosome is still unclear. Some lines of
evidence support the idea that Syf3p is recruited before the formation of complex B, at the time U4/U6.U5 tri-snRNP enters the spliceosome (Chung et al., 1999). Other studies showed that this protein is required for the formation of the active spliceosome, after U1 and U4 snRNA exit (Russell et al., 2000). Syf3p contains 15 tetratricopeptide repeats (TPRs) that serve as a platform for the recruitment of splicing factors.

To date good evidence has been provided to support the presence of Prp45p of S. pombe Snwl or of human SKIP in a multi-protein complex as well as Prp46p and Syf3p. The yeast Cef1p-associated “Cwc” complex contains U2, U5 and U6 snRNAs and more than 20 associated proteins (Ohi et al., 2002). At least eight of them were found associated with the yeast Prp19p in an snRNA-free complex. This protein complex was denominated Prp19-associated or Ntc-complex (Nineteen complex) and is believed to associate with the spliceosome simultaneously or immediately after the dissociation of U4 (Chen et al., 2002). The composition of the yeast Cwc-complex is very conserved to the S. pombe Cwf-complex (Ohi et al., 2002) and to the human 35S U5 snRNP described above (Makarov et al., 2002). It is suggested that these complexes are recruited to the spliceosome prior to the first catalytic step of splicing, remain bound through the process and after the second step when they leave with the excised lariat intron.

Another study performed by Stevens et al. (2002), isolated under mild purification conditions a large penta-snRNPs complex that is able to support pre-mRNA splicing and contains all five snRNAs and associated proteins. Mass spectrometry analysis identified Prp45p, Prp46p, Syf3p as proteins of the yeast penta-snRNPs complex.

1.3.2.2 Role in Transcription

In recent years, many data reported the involvement of the human homologue of Prp45p, NCoA-62/SKIP, in regulation of transcription of different genes. The recent finding of SKIP as a splicing factor argues that this protein and/or its homologues could add an extra link between transcription and splicing.

In 1998 Baudino et al., identified and characterized in mammalian cells a
Vitamin D Receptor, (VDR)-interacting protein, named NCoA-62 which is the homologue of Prp45p. Based on the fact that the interaction of NCoA-62 with VDR augments vitamin D, retinoic acid, estrogen and glucocorticoid-activated transcription, it was considered that NCoA-62 has a nuclear receptor coactivator role within mammalian cells. Other independent studies, a two-hybrid screen isolated NCoA-62 as an interactor with the v-Ski oncoprotein (known to induce both transformation and muscle differentiation depending on the growth conditions); therefore NCoA-62 was renamed Ski-interacting protein or SKIP (Dahl et al., 1998).

Further analyses showed that NCoA-62/SKIP forms a multiprotein complex with VDR-RXR (retinoid X receptor) heterodimer and with SRCs (steroid receptor coactivator) that determine Vitamin D-activated transcription (Zhang et al., 2001). Throughout these interactions, NCoA-62/SKIP contacts the proteins through two different domains located in the highly charged C-terminal domain.

Other studies revealed that SKIP augments Transforming Growth Factor-β (TGF-β)-dependent transcription through its interaction with Smad proteins (Leong et al., 2001). TGF-βs are multifunctional cell-cell signalling proteins that mediate different pathways in growth and development through the activation of Smad proteins and thus trigger the transcription of target genes.

SKIP also interacts with the transcription factor CBF1 that specifically binds to the DNA and represses transcription by tethering a co-repressor complex: Histone Deacetylase Corepressor complex or HDAC. HDAC represses transcription through histone deacetylation, a process that leads to chromatin condensation and inaccessibility of promoter regions to transcriptional factors / RNA Polymerase. SKIP is believed to be a component of this HDAC complex (Zhou et al., 2000a and b).

All the data presented above outline dual action of SKIP on transcription, either stimulatory or inhibitory. Regarding the involvement of other SNW proteins in transcription, Dahl et al., (1998) showed that the Drosophila melanogaster homologue of SKIP, Bx42, was found associated with chromatin in transcriptionally active puffs of salivary glands. Also Bx42 appears to have a role in transcription activation of the steroid hormone (20-OH ecdysone) regulated genes (Wieland et al., 1992).
1.4 This Thesis

The objective of this thesis is to analyse in more detail the function of Prp45p within the splicing machinery in *S. cerevisiae*. To accomplish this, random mutagenesis has been used to render amino acid modifications which might cause a temperature-sensitive phenotype. In order to identify the mutations responsible for the temperature-sensitive phenotype, site-directed mutagenesis has been used to recreate the mutations in the wild-type gene.

From these analyses it appears that mutations within two conserved regions of Prp45p, denominated *A* and *B*, disrupted the protein function at elevated temperatures and determined a mild splicing defect at the second step of splicing.

In addition, the *in vivo* interaction between Prp45p and Prp46p identified in two-hybrid screens has been confirmed *in vitro* by co-immunoprecipitation experiments. Electrophoresis of splicing complexes on native gels has provided evidence that Prp45p is required for the formation of the active spliceosome.
Chapter 2  Materials and Methods
2.1 Materials

2.1.1 General Reagents

Chemicals were purchased from the following sources, except where stated otherwise: Fischer, National Diagnostics, Sigma.

Restriction enzymes, polymerases and other enzymes used in this work were purchased from the following sources except where stated otherwise: Boheringer Mannheim, New England Biolabs., Promega, Qiagen.

Deoxyribonucleotides and ribonucleotides were obtained from Boheringer Mannheim.

Radiochemicals were from Amersham.

Reagents for all growth media were purchased from: Beta Lab, Difco Laboratories, Oxoid and Sigma.

All antibiotics used in this work were from Duchefa.

2.1.2 Sterilisation

Solution and media were typically sterilised by autoclaving for 15 mins. at 120 and 15 lb/inch$^2$. Alternatively, solutions were sterilised by filtration. Small volumes were filtered through acrodisc syringe filters (0.20 μm and 0.45 μm, Sartorius AG). Large volumes of solutions were filtered through 250 ml and 500 ml filter units (0.2 μm and 0.45 μm, Nalgene). Glassware such as corex tubes and glass pipettes were dry sterilised by baking in an oven at 250°C for 16 hrs.
2.1.3 Bacterial and Yeast Growth Media

All growth media were stored at room temperature after autoclaving. For solid media, 2% (w/v) agar was added prior to autoclaving.

<table>
<thead>
<tr>
<th>Table 2.1 Bacterial Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>Luria-Broth (LB)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.2 Yeast Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
</tr>
<tr>
<td>YPDA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>YMM</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>YMM drop-out</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>YMGRSsup</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>YMGRS drop-out</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
2.1.4 Nutrients and supplements (Drop-out mix)

Complete drop-out powder was prepared by mixing 2g of each of the following nutrients: adenine, alanine, arginine asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, tryptophan, uracil, valine, and 4g of leucine. For a specific drop-out powder, the relevant nutrient(s) were omitted.

The drop-out powder was ground to ensure complete mixing. It was added to the media prior to autoclaving.

2.1.5 Antibiotics

Antibiotics were added to liquid media immediately prior to use. For solid media, antibiotics were added after autoclaving. All antibiotic stock solutions were stored at −20°C.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Abbreviation</th>
<th>Solvent</th>
<th>Stock solution (mg/ml)</th>
<th>Final concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>Amp</td>
<td>Water/Ethanol</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
### 2.1.6 Commonly Used Buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X MOPS</td>
<td>1 M MOPS (3-(N-morpholino) propane sulfonic acid</td>
</tr>
<tr>
<td></td>
<td>1 M Tris</td>
</tr>
<tr>
<td></td>
<td>69.3 mM SDS</td>
</tr>
<tr>
<td></td>
<td>20.5 mM EDTA</td>
</tr>
<tr>
<td>20X MES</td>
<td>1 M MES (2-(N-morpholino) ethane sulfonic acid</td>
</tr>
<tr>
<td></td>
<td>1 M Tris</td>
</tr>
<tr>
<td></td>
<td>69.3 mM SDS</td>
</tr>
<tr>
<td></td>
<td>20.5 mM EDTA</td>
</tr>
<tr>
<td>10X PBS</td>
<td>137 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>2.7 mM KCl</td>
</tr>
<tr>
<td></td>
<td>10.1 mM Na$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td>1.76 mM KH$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 7.2</td>
</tr>
<tr>
<td>50X TAE</td>
<td>2 M Tris</td>
</tr>
<tr>
<td></td>
<td>0.05 M EDTA</td>
</tr>
<tr>
<td></td>
<td>5.7% (v/v) Acetic acid</td>
</tr>
<tr>
<td>10X TBE</td>
<td>890 mM Tris</td>
</tr>
<tr>
<td></td>
<td>890 mM Boric acid</td>
</tr>
<tr>
<td></td>
<td>20 mM EDTA</td>
</tr>
<tr>
<td>10X TBS</td>
<td>0.5 M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>1.5 M NaCl</td>
</tr>
</tbody>
</table>
2.1.7 *Escherichia coli* Strains

Strains of bacteria used in this study are listed in Table 2.5. *Escherichia coli* strain TOP10 was routinely used for plasmid cloning and propagation steps.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP10</td>
<td>F'/mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str') endA1 nupG</td>
<td>Grant <em>et al.</em>, 1990</td>
</tr>
</tbody>
</table>
2.1.8 *Saccharomyces cerevisiae* Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMA38a</td>
<td>MATα, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100</td>
<td>M. Albers, This lab.</td>
</tr>
<tr>
<td>BMA64n</td>
<td>MATα, his3-11,15, leu2-3,112, ura3-1, trp1Δ1, ade2-1</td>
<td>M. Albers, This lab.</td>
</tr>
<tr>
<td>YMA45/2</td>
<td>MATα, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, HIS3-Pgal-ProtA:PRP45</td>
<td>M. Albers, This lab.</td>
</tr>
<tr>
<td>YMM45</td>
<td>MATα, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, PRP45-13Myc:TRP1</td>
<td>This thesis</td>
</tr>
<tr>
<td>YMM45-57</td>
<td>MATα, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, prp45-57-13Myc:TRP1</td>
<td>This thesis</td>
</tr>
<tr>
<td>YMM45-113</td>
<td>MATα, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, prp45-113-13Myc:TRP1</td>
<td>This thesis</td>
</tr>
<tr>
<td>YMA151/2</td>
<td>MATα, his3-11,15, leu2-3,112, ura3-1, trp1Δ1, ade2-1, TRP1-Pmet3-HA2:PRP46</td>
<td>M. Albers, This lab.</td>
</tr>
</tbody>
</table>
2.1.9 Oligonucleotides

Oligonucleotides used in this study were purchased from Bioline Ltd. (London), Genosys Biotechnology Ltd. (Cambridge) or MWG-Biotech AG.

<table>
<thead>
<tr>
<th>Table 2.7 Oligonucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
</tr>
<tr>
<td>PS&amp;5-45M</td>
</tr>
<tr>
<td>PNC3-45M</td>
</tr>
<tr>
<td>TNC5-45M</td>
</tr>
<tr>
<td>TP3-45M</td>
</tr>
<tr>
<td>TXHO-45M</td>
</tr>
<tr>
<td>PRP45-F-PROMOTER</td>
</tr>
<tr>
<td>PRP45-R-TERMIN</td>
</tr>
<tr>
<td>PRP45-F1</td>
</tr>
<tr>
<td>PRP45-F2</td>
</tr>
<tr>
<td>Forward</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>F-57-201</td>
</tr>
<tr>
<td>R-57-201</td>
</tr>
<tr>
<td>F-57</td>
</tr>
<tr>
<td>R-57</td>
</tr>
<tr>
<td>F-57-B</td>
</tr>
<tr>
<td>R-57-B</td>
</tr>
<tr>
<td>57B-New-F</td>
</tr>
<tr>
<td>57B-New-R</td>
</tr>
<tr>
<td>F-113</td>
</tr>
<tr>
<td>R-113</td>
</tr>
<tr>
<td>F-113-B</td>
</tr>
<tr>
<td>R-113-B</td>
</tr>
<tr>
<td>F-113-C</td>
</tr>
<tr>
<td>R-113-C</td>
</tr>
<tr>
<td>PRP45-F0</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>TNT-PRP45-46-F</td>
</tr>
<tr>
<td>TNT-PRP45-46-R</td>
</tr>
<tr>
<td>TNT-PRP45-22-New-F</td>
</tr>
<tr>
<td>TNT-PRP45-22-R</td>
</tr>
<tr>
<td>PRP45TNT-5'</td>
</tr>
<tr>
<td>PRP45TNT-3'</td>
</tr>
<tr>
<td>SDM45-Nco-F</td>
</tr>
<tr>
<td>SDM45-Nco-R</td>
</tr>
<tr>
<td>c-Myc-TRP-Nco-F</td>
</tr>
<tr>
<td>c-Myc-TRP-Nco-R</td>
</tr>
<tr>
<td>13-Myc-R</td>
</tr>
<tr>
<td>PRP45-522-F</td>
</tr>
<tr>
<td>PRP45+525R</td>
</tr>
</tbody>
</table>
### Chapter 2: Materials and Methods

#### 2.1.10 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS314</td>
<td>Yeast-E.coli shuttle vector: Multiple cloning site, ( lacZ^\alpha ), Amp(^R), colE1 ori., CEN6, ARSH4, TRP1</td>
<td>Sikorski and Hieter, 1989</td>
</tr>
</tbody>
</table>
## Table 2.8 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBT9</td>
<td>GAL4 DNA binding domain fusion shuttle and expression vector: Multiple cloning site, $Amp^R$, colE1 ori, $P_{ADHI}$, Gal4 DNA-binding domain sequence, $ADHI$ transcriptional terminator, 2μ, $TRP1$.</td>
<td>Clontech Laboratories, Inc.</td>
</tr>
<tr>
<td>pBTM116</td>
<td>LexA DNA-binding domain fusion expression vector: Multiple cloning site, $Amp^R$, colE1 ori, $P_{ADHI}$, LexA sequence, $ADHI$ transcriptional terminator, 2μ, $TRP1$.</td>
<td>S.Fields, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>p283</td>
<td>Modified pGEM1. Contains $ACT1$ coding sequence under regulation of the $T7$ promotor</td>
<td>O’Keefe et al., 1996</td>
</tr>
<tr>
<td>pFA6a-13Myc-TRP1</td>
<td>pFA based <em>E.coli</em> cloning vector carrying a protein-tagging module consisting of 13Myc epitope, the <em>S. cerevisiae ADHI</em> terminator and the <em>S. cerevisiae TRP1</em> marker.</td>
<td>Longtine et al., 1998</td>
</tr>
</tbody>
</table>

## Table 2.9 Modified Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS-PT</td>
<td>Modified pRS314: Two 500 bp fragments representing promoter and terminator regions of <em>PRP45</em> ORF were PCR-amplified from yeast genomic DNA using</td>
<td>This work</td>
</tr>
<tr>
<td>oligonucleotide primers pairs PS&amp;5-45M / PNC3-45M and TNC5-45M / TP3-45M, respectively. The two PCR products were gel-purified, cut with SpeI and NcoI (promoter region) and with NcoI and PstI (terminator region) and then ligated into SpeI, PstI restricted pRS413.</td>
<td></td>
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<tr>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRS-45</td>
<td>Modified pRS314: The PRP45 coding sequence together with its promoter and terminator regions were PCR-amplified from yeast genomic DNA using primers PS&amp;5-45M and TXHO-45M. The PCR product was gel-purified, cut with SpeI and XhoI (restriction sites incorporated into PCR primers) and cloned into the SpeI and XhoI sites of pRS314.</td>
<td></td>
</tr>
<tr>
<td>pRS-45N</td>
<td>Modified pRS-45: The STOP codon of PRP45 coding region TAG was site-directed mutagenesed into ATG using primers SDM45-Nco-F and SDM45-Nco-R. Thus the terminal sequence of the gene CCTAGG became CCATGG; this is an NcoI restriction site.</td>
<td>This work</td>
</tr>
<tr>
<td>Vector</td>
<td>Details</td>
<td>This work</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pMM45</td>
<td>Modified pRS-45N: The 13Myc-TRP1 cassette of pFA6a-13Myc-TRP1 vector was PCR-amplified using primers c-Myc-TRP-Nco-F and c-Myc-TRP-Nco-R. The PCR product was gel purified, cut with NcoI (restriction site incorporated into PCR primers) and ligated into the NcoI restriction site of pRS-45N plasmid. Frame and integrity of the cloned PCR product was checked by sequencing.</td>
<td>This work</td>
</tr>
<tr>
<td>p57A</td>
<td>Modified pRS-45: PRP45 coding sequence was site-directed mutagenized in positions 388 (A to G) and 444 (T to A) using the primers F-57 and R-57.</td>
<td>This work</td>
</tr>
<tr>
<td>p57B</td>
<td>Modified pRS-45: PRP45 coding sequence was site-directed mutagenized in positions 623 (T to C) and 628 (A to G) using the primers F-57-B and R-57-B.</td>
<td>This work</td>
</tr>
<tr>
<td>p57AB</td>
<td>Modified p57A: PRP45 coding sequence in p57A vector was site-directed mutagenized in position 623 (T to C) using primers 57B-New-F and 57B-New-R.</td>
<td>This work</td>
</tr>
<tr>
<td>p57-201</td>
<td>Modified pRS-45: PRP45 coding sequence was site-directed mutagenized in position 574 (A to G).</td>
<td>This work</td>
</tr>
<tr>
<td>p113A</td>
<td>Modified pRS-45: PRP45 coding sequence was site-directed mutagenized in positions 388 (A to G) and 397 (A to G) using the primers F-113 and R-113.</td>
<td>This work</td>
</tr>
<tr>
<td>p113B</td>
<td>Modified pRS-45: PRP45 coding sequence was site-directed mutagenized in position 33.</td>
<td>This work</td>
</tr>
<tr>
<td><strong>p113AB</strong></td>
<td>Modified p113A: <strong>PRP45</strong> coding sequence in p113A vector was site-directed mutagenized in position 611 (T to C) using primers F-113-B and R-113-B.</td>
<td>This work</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>p113C</strong></td>
<td>Modified pRS-45: <strong>PRP45</strong> coding sequence was site-directed mutagenized in position 818 (T to C) using the primers F-113-C and R-113-C.</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pMM57</strong></td>
<td>Modified p57AB: A 2300 bp fragment containing c-terminus of <strong>PRP45</strong> ORF, the 13Myc-TRP1 cassette and 500 bp <strong>PRP45</strong> terminator was isolated from pMM45 via SfoI, XhoI restriction. The fragment was ligated into SfoI, XhoI digested p57AB. The identity of the cloned fragment was verified by sequencing.</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pMM113</strong></td>
<td>Modified p113AB: A 2300 bp fragment containing c-terminus of <strong>PRP45</strong> ORF, the 13Myc-TRP1 cassette and 500 bp <strong>PRP45</strong> terminator was isolated from pMM45 via SfoI, XhoI restriction. The fragment was ligated into SfoI, XhoI digested p113AB. The cloned fragment was checked by sequencing.</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pMA45</strong></td>
<td>Modified pBTM116: <strong>PRP45</strong> coding sequence was fused to <em>LexA</em> DNA-binding domain. <strong>PRP45</strong> ORF was cloned into the EcoRI and SalI sites of pBTM116.</td>
<td>M. Albers, This lab.</td>
</tr>
<tr>
<td>pGBT9/SKIP</td>
<td>Modified pGBT9: The SKIP coding sequence was fused to the GAL4 DNA-binding domain sequence in the multiple cloning site of pGBT9.</td>
<td>Mike Hayman, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------------------------------------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>pGBT9/SKIP</td>
<td>Modified pGBD9: The sequence encoding amino acids 1-200 of SKIP was fused to the GAL4-DNA binding domain sequence in the multiple cloning site of pGBT9.</td>
<td>J. Figueroa, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>(1-200)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGBT9/SKIP</td>
<td>Modified pGBD9: The sequence encoding amino acids 1-300 of SKIP was fused to the GAL4-DNA binding domain sequence in the multiple cloning site of pGBT9.</td>
<td>J. Figueroa, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>(1-300)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGBT9/SKIP</td>
<td>Modified pGBD9: The sequence encoding amino acids 201-537 of SKIP was fused to the GAL4-DNA binding domain sequence in the multiple cloning site of pGBT9.</td>
<td>J. Figueroa, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>(201-537)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGBT9/SKIP</td>
<td>Modified pGBD9: The sequence encoding amino acids 334-537 of SKIP was fused to the GAL4-DNA binding domain sequence in the multiple cloning site of pGBT9.</td>
<td>J. Figueroa, S.U.N.Y., Stony Brook</td>
</tr>
<tr>
<td>(334-537)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.11 Antisera

The antisera used in this study are listed in Table 2.10.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HA</td>
<td>Mouse monoclonal antibody (clone 12CA5) raised against the influenza hemagglutinin protein. 1: 1,000 dilution for Western blotting. 4 μl for immunoprecipitation.</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-c-Myc (sc-40)</td>
<td>Mouse monoclonal antibody raised against the human c-Myc COOH terminus. 1:1,000 for Western blotting.</td>
<td>SantaCruz Biotechnology</td>
</tr>
<tr>
<td>Anti-c-Myc (sc-789)</td>
<td>Rabbit polyclonal antibody raised against the human c-Myc COOH terminus. 1:1,1000 for Western blotting.</td>
<td>SantaCruz Biotechnology</td>
</tr>
<tr>
<td>Anti-Mouse IgG-HRP</td>
<td>Sheep Anti-mouse IgG covalently linked to horseradish peroxidase. 1:1,000 dilution for Western blotting.</td>
<td>Amersham</td>
</tr>
<tr>
<td>Anti-Rabbit IgG-HRP</td>
<td>Donkey Anti-rabbit IgG covalently linked to horseradish peroxidase. 1:1,000 dilution for Western blotting.</td>
<td>Amersham</td>
</tr>
</tbody>
</table>
2.2 **Microbiological Methods**

2.2.1 Growth of Bacteria

*E. coli* strains were grown in LB medium at 37°C. To maintain selection for a plasmid, transformed bacteria were grown in medium containing the appropriate antibiotic (Ampicillin in this work) (see Table 2.3).

2.2.2 Growth of Yeast

Yeast strains were routinely grown at 30°C (or at 23°C for temperature-sensitive strains) in YPDA or YMGRSsup (Table 2.2). To maintain selection for plasmid DNA or to select transformants for integration of a reporter gene into the genome, cells were grown in YMM or YMGRS with appropriate dropout powder added to the medium (YMM –X or YMGRS –X; Table 2.2).

2.2.3 Preservation of Bacterial Strains

*E. coli* strains were stored for up to two weeks at 4°C. To preserve strains indefinitely, bacteria were grown in appropriate medium to stationary phase. One millilitre of culture was mixed with an equal volume of sterile 30% (w/v) glycerol, so that the final concentration is 15% (w/v) glycerol. This was subsequently snap-frozen on dry ice and stored at -70°C.

2.2.4 Preservation of Yeast

*S. cerevisiae* strains were stored for up to two months on solid medium at 4°C. To preserve strains indefinitely, yeast cells were grown in appropriate medium to mid-
logarithmic phase. One millilitre of culture was mixed with an equal volume of sterile 30\% (w/v) glycerol, so that the final concentration is 15\% (w/v) glycerol. This was subsequently snap-frozen on dry ice and stored at -70°C.

2.2.5 Transformation of *E. coli*

2.2.5.1 Preparation of Electro-competent Cells

TOP10 *E. coli* cells were taken from -70°C stock and grown overnight at 37°C on solid LB medium (Table 2.1). A single colony was inoculated in 10 ml of LB liquid medium and incubated overnight at 37°C. An aliquot of this culture was used to inoculate 500 ml of LB liquid medium to an optical density at 600 nm (OD$_{600}$) of 0.1 units. The culture was grown at 37°C until the OD$_{600}$ was 0.5-0.7. The cells were placed on ice for 10 minutes. From this stage, all the manipulations were done on ice using pre-chilled solutions and containers. Cells were sedimented for 20 minutes at 4,200 rpm (2°C, Beckman JLA 10.500 rotor), then they were washed once 500 ml and once in 250 ml of sterile, distilled water. The cells were then washed in 10 ml of 10\% (v/v) glycerol and sedimented as before. Finally, cells were resuspended in 1 ml of 10\% (v/v) glycerol and aliquoted to 40μl on ice. The competent cells were snap frozen on dry ice and stored at -70°C indefinitely.

2.2.5.2 Transformation of Electro-competent Cells

Electro-competent TOP10 *E.coli* cells were thawed on ice and mixed with 100 ng of transforming DNA. Cells were transferred to a pre-chilled electroporation cuvette (0.2 cm electrode gap; Equibio) on ice. Residual ice and water from the surface of the cuvette was wiped off with a tissue paper. To remove the trapped air between the electrodes and to ensure the cells are at the bottom, the electrocuvette was taped few times onto the bench. Electroporation was performed using a Biorad Gene Pulser II at 200 ohms resistance, 25 μF capacity and 2.5 KV voltage. Immediately upon electroporation, 1 ml of LB (Table 2.1) was added and the cells transferred to a sterile Eppendorf tube. The transformed cells were allowed to recover for 1 hour, at
37°C, on a rotating wheel. The cells were plated on solid LB medium supplemented with the appropriate antibiotic to ensure the maintenance of the transformed plasmid. The plates were incubated overnight at 37°C.

### 2.2.6 Transformation of Yeast

A single colony of yeast strain to be transformed was grown overnight into 10 ml of appropriate liquid medium (Table 2.2) at 30°C. The cells were then diluted into 50 ml of fresh medium to an OD\textsubscript{600} of 0.1 and this was further incubated till the OD\textsubscript{600} reached 0.5 – 0.7. The cells were sedimented at 3,500 rpm (MSE Mistral 1000 centrifuge) for 5 minutes, washed once in 50 ml of sterile, distilled water and harvested as above. The cell pellet was resuspended in 25 ml of sterile LiT solution supplemented with 0.25 ml of 1M DTT and incubated for 40 minutes at room temperature with gentle shaking. Following this incubation, the cells were sedimented by centrifugation as before, and resuspended in 0.75 ml LiT / 7.5 μl 1M DTT. One hundred microlitres of cell suspension was then added to a mixture of: 50 μl LiT, 1-10 μl of transforming DNA (0.1-2 μg/μl) and 50 μg of single stranded salmon sperm carrier DNA (incubated at 100°C for 10 minutes prior to use). The samples were incubated at room temperature for 10 minutes. Three hundred microlitres of freshly prepared PEG solution was added and the incubation continued for another 10 minutes. Subsequently, 50 μl of DMSO was added and the cells were heat-shocked by transferring the tubes into a water bath at 42°C for 15 minutes. Then the cells were sedimented for 1 minute at 4,000 rpm, the supernatant removed and the pellet resuspended in 1 ml of appropriate media. The transformed cells were allowed to recover for 1 hour, at 30°C, on a rotating wheel. The cells were harvested by centrifugation (4,000 rpm, 60 seconds) and then resuspended in 400 μl of sterile, distilled water. Finally, 100 μl aliquots were spread onto the appropriate selective solid media (Table 2.2). The plates were incubated at 30°C (23°C for temperature-sensitive mutants) for 3-4 days.
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- LiT: 10 mM Tris-Cl (pH 7.5), 100 mM Lithium acetate
- PEG: 100% (w/v) PEG4000, 10 mM Tris-Cl (pH 7.5), 100 mM Lithium acetate

2.2.7 Transformation and Genomic Integration of Temperature-sensitive Alleles

A linear DNA fragment containing the temperature-sensitive allele and a cassette containing TRPI gene as a marker and a sequence encoding for 13 Myc epitope was generated by PCR. The PCR was performed using plasmid DNA as a template. The final PCR product has at least 200 bp of sequence identical to the region immediately upstream and downstream of the ORF to be replaced. The PCR product was gel-purified from the template plasmid DNA and approximately 4μg were transformed into haploid yeast cells of strain BMA38a (Table 2.6). Following the 42°C heat-shock step, the transformed cells were incubated for 3 hours at 23°C on rotating wheel. The cells were spread onto medium lacking tryptophan (-Trp) to select for TRPI marker and the plates incubated for up to 4 days at 23°C. Colonies growing on these plates were streaked onto fresh solid medium and the integration was investigated using PCR, Western blotting and sequencing.

2.2.8 Growth Curves

Growth curve analysis was performed on yeast cells that were temperature-sensitive. Cells were grown in liquid media to mid-logarithmic phase under permissive conditions (23°C). Then aliquots of culture were used to inoculate pre-warmed medium to an OD_{600} of 0.05 – 0.1. Cultures were then incubated at the appropriate temperature with the growth rate monitored by measuring the OD_{600} at regular intervals, typically every hour. In order to keep all OD_{600} readings below 0.7 and thus maintain the cells in logarithmic growth, the cultures were diluted with pre-warmed growth medium.
2.2.9 β-Galactosidase Assay

This protocol is modified after the one described by Legrain and Rosbash, 1989. The yeast strains of interest were transformed with the reporter plasmids (containing \textit{URA3} marker) and the transformants plated on medium lacking uracil (-Ura). Five milliliters of GGL-U media were inoculated with a single colony and grown at 23°C. When the $\text{OD}_{600}$ reached 0.5, 2% (v/v) of galactose was added and the cultures grown for 2 hours at non-permissive temperature (37°C). Then, aliquots of 1 ml were removed, the cells were spun for 3 minutes at 14,000 rpm and resuspended in 0.5 ml of Buffer Z (supplemented with 0.36% (v/v) of β-mercaptoethanol). Two hundred microliters of ether was added and the cells were vortexed for 20 seconds. The lysates were spun for 1 minute at room temperature to separate the phases and the ether let to evaporate. The β-galactosidase reaction was started by adding 100 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/ml in Buffer Z) and incubated at 30°C. When a pale yellow colour developed, the reactions were stopped with 250 μl of 1M Na$_2$CO$_3$. The reactions were centrifuged for 5 minutes at 14,000 rpm and the optical densities were read at 420 nm. The β-galactosidase activity was expressed in units according to the formula:

$$\text{β-galactosidase activity (units)} = 1000 \times \frac{\text{OD}_{420}}{\text{OD}_{600} \times \text{Vol} \times \text{Time}}$$

where:

- \text{“OD}_{420}” is the absorbance read from the sample at 420 nm;
- \text{“OD}_{600}” is the turbidity of cells read 2 hours after galactose induction;
- \text{“Vol”} is the volume of culture used for the assay (i.e. 1 ml);
- \text{“Time”} is the time of reaction in minutes.
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GGL-U media: 0.067% (w/v) Yeast Nitrogen Base w/o amino acids
   2% (w/v) glycerol
   2% (w/v) lactate, pH 5.7
   0.05% (w/v) glucose
   0.2% (w/v) Ura' drop-out powder (section 2.1.4)
   pH adjusted with 1 pellet of NaOH per 500 ml

Buffer Z: 100 mM Na$_2$HPO$_4$, pH 7.5
   10 mM KCl
   1 mM MgSO$_4$
2.3 Nucleic Acids Methods

2.3.1 General Methods

2.3.1.1 Extraction with phenol:chloroform:isoamyl alcohol

To purify the nucleic acid solution from proteins an equal volume of phenol:chloroform:isoamyl alcohol (P/C/I) (25:24:1) was added, vortexed for 10 seconds and centrifuged at 14,000 rpm for 5 minutes. The upper (aqueous) phase, containing the nucleic acids was carefully removed into a fresh Eppendorf tube.

2.3.1.2 Precipitation of nucleic acids

Nucleic acids were precipitated from aqueous solution by mixing with 1/10 volume of 3 M NaOAc (pH 5.2) and 3 volumes of ice-cold ethanol (abs.) and freezing at -70°C for at least 1 hour. Nucleic acids were pelleted by centrifugation at 14,000 rpm for 20 minutes at 4°C and then washed with ice-cold 70% ethanol and spin as before. The supernatant was removed and the pellet dried under vacuum, resuspended in the appropriate volume of distilled sterile water and, where applicable, the nucleic acid concentration was determined.

2.3.1.3 Spectrophotometric Quantification of Nucleic Acids

The concentration of DNA or RNA was determined by measuring the absorption of diluted solutions (usually 1:1000) at a wavelength of 260 nm using the Cecil CE 2040 spectrophotometer. The measurements were done in quartz cuvette. For double stranded DNA an OD_{260} value of 1.0 represents a DNA concentration of 50 μg/ml. For single-stranded RNA an OD_{260} of 1.0 represents RNA concentration of approximately 40 μg/ml. The calculations were performed using the online DNA/RNA calculator at:
http://www.geocities.com/CapeCanaveral/Lab/9965/calculator-dnaconc.html. To determine if the nucleic acid solution was protein free, the absorption at wavelengths of 260 nm and 280 nm were measured. Pure preparation of DNA / RNA solution should give an \( \text{OD}_{260} : \text{OD}_{280} \) ratio value between 1.8 and 2.0.

2.3.2 DNA Methods

2.3.2.1 Small Scale Preparation of Plasmid DNA from by Spin Column

Plasmid DNA from *E.coli* was prepared using the QIAprep kit (Quiagen) following the manufacturer’s instructions. DNA was extracted from 3 ml of *E. coli* cell culture grown to stationary phase, eluted with 50 μl of distilled water and stored at -20°C.

2.3.2.2 Plasmid Rescue from Yeast Cells

A single yeast colony containing a plasmid was collected with a toothpick and resuspended in an Eppendorf tube with 200 μl of lysis buffer. Two hundred microlitres of glass beads (150-212 μm in diameter; Biospec Products Inc.) and 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1) were added and the tube vortexed for 5 minutes and then subjected to centrifugation (14,000 rpm, 5 minutes). The upper phase was removed and mixed with 1/2 volume of 7.5M ammonium acetate (NH₄OAc) and 4 volumes of ice-cold ethanol (abs.) and placed on dry-ice for 20 minutes. The DNA was pelleted by centrifugation at 14,000 rpm for 10 minutes at 4°C then washed with 70% (v/v) ethanol and vacuum dried. The pellet was resuspended in 10 μl of sterile distilled water. Subsequently, 1.5 μl was transformed in electro-competent *E. coli* cells and the transformants plated onto the appropriate media (section 2.2.5). A single *E. coli* colony was collected and plasmid DNA was extracted (section 2.3.2.1).
Lysis buffer: 2% (v/v) Triton X-100
1% (w/v) SDS
100 mM NaCl
10 mM Tris-HCl, pH 8.0
1 mM EDTA

2.3.2.3 Preparation of Yeast Genomic DNA

Yeast genomic DNA was prepared using the method of Hoffman and Winston, 1987. Ten milliliters of the appropriate selective medium were inoculated with a single yeast colony and grown overnight at the appropriate temperature in a shaking incubator. The cells were harvested by centrifugation at 3,500 rpm for 5 minutes (MSE Mistral 1000 centrifuge) and then washed in 10 ml of sterile, distilled water. The cells were resuspended in 1 ml of sterile, water and transferred to an Eppendorf tube, pelleted at 14,000 rpm for 10 seconds. Two hundred microlitres of lysis buffer (section 2.3.2.2), 200 μl of glass beads (150-212 μm) and 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1) were added and the mixture vortexed for 4 minutes. Two hundred microlitres of sterile, distilled water was added, the tube vortexed for 10 seconds and then centrifuged (14,000 rpm, 5 min, RT). The upper, DNA containing phase was removed and transferred into a fresh Eppendorf tube. Then the DNA was precipitated (see section 2.3.1.2), the pellet vacuum dried and resuspended in 100 μl of distilled water. Typically, a yield of 0.2 μg/μl was obtained.

2.3.2.4 Restriction Digestion of DNA

Restriction endonuclease digestion of DNA was typically performed in volumes of 20-50 μl. These volumes contain the required quantity of DNA (usually 2-5 μg) and the appropriate restriction buffer (as supplied by the manufacturer) at 1X concentration. The restriction enzyme (typically 2-5 units) was added the last to the reaction taking care to keep the restriction enzyme volume below 10% of the total
volume. The restriction digest was incubated at the temperature recommended by the supplier for a period of 2-10 hours. For the double digest reaction, both enzymes were added concurrently providing that both would retain full activity in a suitable buffer. The products of the digestion were analysed by agarose gel electrophoresis (see section 2.3.2.8) and isolated from the gel and purified (as described in section 2.3.2.10).

2.3.2.5 Removal of 5' Phosphates from DNA

Plasmid DNA digested with restriction endonucleases (section 2.3.2.4) was incubated with 5 units of Calf Intestinal Alkaline Phosphatase (CIP) (for 20 µl reaction) for 30 minutes at 37°C. In this way the terminal phosphate groups were removed and therefore the recircularisation of the vector DNA in any subsequent ligation reactions (see section 2.3.2.11).

2.3.2.6 Amplification of DNA using the Polymerase Chain Reaction

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). A PCR reaction mix was set-up in a 0.5 ml Eppendorf tube as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Polymerase Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10-500 ng of yeast genomic DNA or 10-30 ng of plasmid DNA</td>
</tr>
<tr>
<td>Oligonucleotid primer 1</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Oligonucleotid primer 2</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>dNTPs (dATP, dCTP, dGTP, dTTP)</td>
<td>200 µM each</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5-6 mM</td>
</tr>
<tr>
<td>DNA polymerase*</td>
<td>1.25 units per 50 µl</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>up to 50 µl</td>
</tr>
</tbody>
</table>

PCR reactions were carried in a PTC-100 Hot Lid reactor (MJ Research)
programmed depending on the length of the desired product and the annealing temperature of oligonucleotide primers used.

A typical program contained the following steps:

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Denaturation</td>
<td>94°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Step 2</td>
<td>Denaturation</td>
<td>94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 3</td>
<td>Annealing</td>
<td>$T_d^b$°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 4</td>
<td>Extension</td>
<td>72°C</td>
<td>1 minute$^c$</td>
</tr>
<tr>
<td></td>
<td>30 cycles of Step 2 - Step 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 5</td>
<td>Final extension</td>
<td>72°C</td>
<td>3 minute</td>
</tr>
</tbody>
</table>

$^a$for most applications, Taq DNA polymerase was used. When an accurate amplification of the DNA was needed (i.e. cloning of PCR products for gene expression), Pfu polymerase was used.

$^b$$T_d^b$°C is the annealing temperature calculated for oligonucleotides up to 20 bases long with the formula:

$$T_d^b = \frac{4(G+C) + 2(A+T)}{4}$$

$^c$One minute extension time per every kb of the length of the desired product.

2.3.2.7 PCR from Yeast Colonies

One large yeast colony was picked with a sterile toothpick and resuspended in 10 μl of freshly prepared 0.02 M NaOH. The cell suspension was briefly vortexed and boiled for 5 minutes and immediately put on ice until needed. Two microliters of cell suspension were added to a 30 μl PCR reaction assembled on ice. The PCR conditions are as above, except that the thermocycler was pre-heated to 94°C before the program took place.
2.3.2.8 Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA fragments produced by restriction endonuclease digest (section 2.3.2.4) or generated by PCR (section 2.3.2.6) was typically performed in 0.8-1% (w/v) agarose gels. Gels were prepared by melting the appropriate amount of agarose in 1x TAE buffer (Table 2.4) and adding ethidium bromide to a final concentration of 0.1 μg/ml. DNA samples to be analysed were mixed with 1X Agarose gel loading buffer and loaded directly onto the gel. The gel was placed in 1X TAE buffer and run at a constant voltage of 150-200V. To estimate the size of DNA fragments, 1Kb Plus DNA Ladder (Gibco BRL) was also loaded on the gel. The DNA could be visualised on a UV transilluminator.

10X Agarose gel loading buffer: 0.25% (w/v) Bromphenol blue
30% (v/v) Glycerol

2.3.2.9 Purification of PCR Products

If the PCR performance generated a single product (as seen on agarose gel), this was purified from oligonucleotide primers, unincorporated nucleotides, polymerises and salts using the QIAquick PCR purification kit (Quiagen) according to manufacturer’s instructions. The DNA was eluted in 30 μl of elution buffer (also supplied in a kit) and stored at -20°C.

2.3.2.10 Purification of DNA from Agarose Gels

As an alternative to the previous protocol, the DNA fragments produced by PCR or by restriction endonuclease digestion were purified after separation on an agarose gel. Using a clean razor blade, the band of interest was excised from the gel and further purified using the QIAquick gel extraction kit (Qiagen), following the manufacturer’s guidelines. DNA was typically eluted in 30 μl of elution buffer.
(supplied with the kit) and stored at -20°C.

2.3.2.11 Ligation of DNA Molecules

Ligations were typically performed in a total volume of 15 µl, containing 20-30 ng of vector DNA, approximately 8 times this amount of insert DNA, 1X ligation buffer, 1mM ATP and 2 units of Fast-Link DNA ligase (Epicentre Technologies). The reactions were incubated at room temperature overnight and stopped by heat-inactivation of the enzyme for 15 minutes at 70°C. The reaction mix was ethanol precipitated, the pellet washed (section 2.3.1.2) and resuspended in 1.3 µl of sterile, distilled water. The whole amount was used to transform 40 µl of E.coli competent cells (section 2.2.5).

2.3.2.12 Mutagenic PCR of PRP45 gene using skewed nucleotide concentrations

This protocol is modified after Leung et al., 1989.

In order to randomly mutagenized PRP45 gene, the same protocol for PCR amplification of plasmid DNA was used except that the concentration of one deoxynucleotide (dATP) was lower.

The mutagenic PCR reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Taq DNA Polymerase buffer</td>
<td>1X</td>
</tr>
<tr>
<td>Plasmid DNA (pRS-45)</td>
<td>15 ng</td>
</tr>
<tr>
<td>DMSO</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>6 mM</td>
</tr>
<tr>
<td>Direct primer (PS&amp;5-45M)</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Reverse primer (TXHO-45M)</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>dGTP, dCTP, dTTP</td>
<td>1 mM</td>
</tr>
<tr>
<td>dATP</td>
<td>200 µM</td>
</tr>
<tr>
<td>sterile, distilled water</td>
<td>up to 50 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>5U</td>
</tr>
</tbody>
</table>
The PCR product was gel-purified (section 2.3.2.10), resuspended in 30 μl of sterile water and then quantified by running 10% of the total volume on agarose gel. Five hundred nanograms of the purified PCR product were used for transformation.

2.3.2.13 Site-directed mutagenesis

Two methods of side-directed mutagenesis have been used in this thesis: 1) site directed mutagenesis by overlap extension and 2) site-directed mutagenesis by whole plasmid synthesis. Both protocols allow PCR (section 2.3.2.6) to be used for introducing site-directed mutations into PRP45. The PCR was carried out on miniprep plasmid DNA as a template (section 2.3.2) and using mutagenic primers containing desired mutations.

2.3.2.13.1 Site-directed mutagenesis by overlap extension

This protocol is adapted after the one described by Ho et al., 1989. Two separate PCRs were performed on the template wild-type PRP45 gene to give products T2 and T3 (Figure 2.1). The primers used P1 and P2 (for T2) and P3 and P4 (for T3) are designed as follows:

- P2 and P3 overlap and contain the desired mutations in the middle with 10-15 bases of correct sequence on either side;
- P1 (PS&5-45M or S) and P4 (TXHO-45M or X) have added restriction sites at 5' ends SpeI and XhoI, respectively, so that the final product, T1 can be ligated into the original vector that contained the wild-type gene (pRS-45).

The PCRs were carried out as described in section 2.3.2.6 with the following specifications:

- the amount of primers, template and dNTPs was 2.5X higher than for the normal PCR;
- in order to reduce mis-incorporations, there were 15 rounds of amplification instead of 30.
Figure 2.1 Site-directed mutagenesis of \textit{PRP45} by overlap extension.

Templates T2 and T3 were run on agarose gel to check their sizes and gel-purified (section 2.3.2.10). The final product T1 containing \textit{prp45} allele was made by hybridizing the overlapping strands from the two fragments (around 50 ng each) and extending this overlap using a normal PCR protocol and the primers P1 and P4. Finally, T1 was gel-purified, digested with the restriction enzymes \textit{Spel} and \textit{XhoI} and
ligated into the original plasmid (section 2.3.2.11).

2.3.2.13.2 Site-directed mutagenesis by whole plasmid synthesis

This method is adapted after the one described by Weiner et al. (1994). The DNA template (pRS-45 plasmid) was denatured before being subjected to PCR. Ten micrograms of plasmid miniprep was treated with 10 μl of Denaturing solution in a total volume of 50 μl and incubated for 15 minutes at 37°C. The DNA was precipitated according to the protocol described in section 2.3.1.2 and resuspended in 20 μl of sterile water. Two microliters of denatured pRS-45 were used for the PCR reaction in a final volume of 50 μl. The oligonucleotide primers (0.5 μM), each complementary to opposite strand of the vector and containing the desired mutation(s), are extended during temperature cycling by *Pfu* DNA polymerase.

The PCR profile used is the following:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1 – Denaturation:</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 2 – Denaturation:</td>
<td>95°C</td>
<td>0.5 minute</td>
</tr>
<tr>
<td>Step 3 – Annealing:</td>
<td>Td</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 4 – Extension:</td>
<td>68°C</td>
<td>2 minutes / kb</td>
</tr>
<tr>
<td>18 cycles of Step 2 – Step 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 5 – Final extension</td>
<td>68°C</td>
<td>10 minute</td>
</tr>
</tbody>
</table>

Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks (Figure 2.2). The PCR product was checked on agarose gel.
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Figure 2.2 Site-directed mutagenesis by whole plasmid synthesis.
Then 20U of \textit{DpnI} was added directly to the PCR reaction and the digestion was incubated for 2 hours at 37°C. \textit{DpnI} restriction enzyme digests the non-mutated parental DNA template and selects for the synthesized DNA containing mutations. Since DNA isolated from most \textit{E. coli} strains is dam methylated, it is susceptible to \textit{DpnI} digestion, which is specific for methylated and hemimethylated DNA. The nicked vector DNA incorporating the desired mutations is then transformed into TOP 10 \textit{E. coli} strain. After transformation, the \textit{E. coli} cells repair the nicks in the mutated plasmid.

Denaturing solution:  
1M NaOH  
1mM EDTA, pH 8.0

2.3.2.14 End-labelling of Oligonucleotides

For Northern blot analysis oligonucleotides were labelled in a 20 μl reaction using T4 polynucleotide kinase (PNK, New England Biolabs). The reaction mix was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotide (10pmol/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>10X PNK buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>([γ-^{32}P]ATP) (~5000 Ci/mmol)</td>
<td>2.0</td>
</tr>
<tr>
<td>T4 PNK (10 units/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td>Sterile, distilled water</td>
<td>14.5</td>
</tr>
</tbody>
</table>

The labelling reaction was incubated at 37°C for 30 minutes. Prior to blotting, the radiolabelled oligonucleotide reaction was purified by passing through a 2 μm filter.
2.3.2.15 DNA Sequencing

Plasmid DNA to be sequenced was prepared using the QIAprep spin columns (section 2.3.2.9) and quantified by visualisation on an agarose gel (section 2.3.2.8). For genomic DNA, the segment to be sequenced was first amplified by PCR (section 2.3.2.6), purified using QIAquick PCR purification kit and 10% of the eluted product was quantified by visualisation on agarose gel. Sequencing reactions were performed with the BigDye Terminator Cycle Sequencing Ready Reaction in a PTC-100 Hot Lid reactor (Genetic Research Instrumentation Ltd). A typical reaction mix was set up as follows:

- **Template DNA**: 200 ng of plasmid DNA or 100-120 ng of PCR product
- **Primer**: 1.6 pmol
- ** Terminator mix**: 4 µl
- **Sterile, distilled water**: up to 10 µl

The following sequencing program was run for 25 cycles:

- **Step 1**: 96°C for 30 seconds
- **Step 2**: 50°C for 15 seconds
- **Step 3**: 60°C for 4 minutes

The DNA was precipitated from the reaction mix by adding 25 µl of ice-cold ethanol (abs.) and 1 µl of 3 M NaOAc, pH 5.2, incubating on ice for 20 minutes followed by centrifugation (13,000 rpm, 30 minutes, 4°C). The pellet was washed with 250 µl of ice-cold 70% (v/v) ethanol and dried on bench for 1 hour. Alternatively, the sequencing reactions were purified on genCLEAN Dye Terminator Removal Columns (GENETIX), according to the producer’s instructions. The samples were run by Jill Lovell (ICAPB, University of Edinburgh) on an ABI
PRISM® 377 DNA Sequencer and the resulting sequences were analysed using Sequencer TM4 computer program.

2.3.3 RNA Methods

2.3.3.1 Total RNA Preparation from Yeast

Yeast total RNA was prepared using the modified protocol of Schmitt et al., 1990.

Fifty millilitres of the appropriate medium (Table 2.2) was inoculated with a single colony of yeast strain and grown overnight at the suitable temperature. Upon reaching OD$_{600}$ of 0.5-0.7, the cells were sedimented by centrifugation at 3,500 rpm for 5 minutes (MSE Mistral 1000 centrifuge). The supernatant was removed and the cells resuspended in 400 µl of AE buffer. At this point the cells could be stored in AE buffer for 6 months at -70°C. Otherwise, 50 µl of 10% (w/v) SDS and 500 µl of phenol-AE were added to the cells, vortexed twice vigorously and incubated at 65°C for 4 minutes. The samples were snap-frozen in liquid nitrogen and allowed to thaw before being centrifuged at 14,000 rpm for 5 minutes. The aqueous phases was removed and extracted twice with phenol-AE: chloroform (1.2:1) and once with ice-cold chloroform. The aqueous layer was again removed in an Eppendorf and precipitated with ethanol (section 2.3.1.2). The precipitated RNA was dried under vacuum and resuspended in 40 µl of ice-cold DEPC-treated water and the concentration measured at spectrophotometer (section 2.3.1.3).

AE buffer: 50 mM NaoAc, pH 5.2
10 mM EDTA, pH 8

Phenol-AE: Phenol equilibrated with AE buffer in a ratio of 4:1 and left to get separated overnight at 4°C.
2.3.3.2 RNA Gel Electrophoresis and Northern Blot Analysis

Low molecular weight RNAs (shorter than 600nt) were separated on polyacrylamide-urea gels. The gels (6%) were prepared by mixing 20 ml Sequagel-6 and 5 ml Sequagel complete buffer reagent (National Diagnostics), as well as adding 300 µl of 10% (w/v) ammonium persulphate (APS). 10 µg of yeast total RNA (section 2.3.3) was mixed with three volumes of Sanger loading dye and heated to 95°C for 3 minutes immediately prior to loading. Gels were run in 1X TBE buffer (Table 2.4) at room temperature (24W, 1 hour).

The RNA was then transferred electrophoretically from the gel to a Hybond™-N nylon membrane (Amersham Pharmacia) in 0.5X TBE (Table 2.4) for 3 hours at 50V. Once the transfer complete, the membrane was blotted dry and the RNA was cross-linked to the membrane in a Stratagene UV Stratalinker using the 'autocrosslink' function to immobilise the RNA on the filter.

Sanger loading dye: 51% (v/v) Formamide
20 mM EDTA
0.3% (w/v) Xylene xyanol
0.3% (w/v) Bromophenol blue

High molecular weight RNAs (longer than 600nt) were separated on agarose gels. Agarose gels (2%(w/v)) were prepared by melting 4g of agarose in 200 ml of 0.5X TBE buffer (Table 2.4). Total RNA (8 - 10µg) prepared as described in section 2.3.3 was mixed with 5 volumes of RNA loading buffer and heated to 65°C for 15 minutes immediately prior to loading. Electrophoresis was performed in 0.5X TBE buffer at 60V for approximately 16 hours.
RNA loading buffer: 50% formamide  
6% formaldehyde  
0.1X HEPES  
10% (v/v) glycerol  
0.025% (w/v) bromphenol blue  
0.025% (w/v) xylene cyanol  
0.01% (w/v) ethidium bromide

The agarose gel was washed twice in distilled water, 5 minutes each, with gentle agitation. The RNA was transferred in 6X SSC (Table 2.4) by capillary force to a Hybond™-N nylon membrane (Amersham Pharmacia). Transfer was allowed to take place overnight and, following this, the RNA was immobilised on the membrane by cross-linking as above.

2.3.3.3 Hybridisation of Northern Blots

The membranes were hybridised with 5’end labelled oligonucleotides (section 2.3.2.14). Two protocols of hybridisation were followed depending on the length of the radiolabelled oligonucleotide used.

a) End-labelled oligonucleotides smaller than 35 nucleotides were hybridised as follows: the nylon membrane was pre-hybridised in 50 ml of hybridisation buffer-S (pre-warmed to 65°C) for at least one hour at 37°C. The labelled probe was added to the membrane and the incubation continued overnight at 37°C. The following morning the probe was decanted off and stored at -20°C for possible re-use. The membrane was washed twice for 20 minutes in 6X SSPE buffer (Table 2.4) pre-warmed to 42°C. Following washings, the nylon membrane was blotted dry, wrapped in Saran wrap to prevent it drying out and subjected to autoradiography at -70°C.
b) For end-labelled oligonucleotides larger than 35 nucleotides, the pre-hybridisation was done in formamide hybridisation buffer (pre-warmed to 65°C) for at least one hour at 37°C. The protocol was similar to a) with the exception of the washings, which were done with 2X SSPE (Table 2.4).

<table>
<thead>
<tr>
<th>Hybridisation buffer-S:</th>
<th>10X SSPE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5X Denhardt's solution</td>
</tr>
<tr>
<td></td>
<td>0.5% (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>200 μg/ml Salmon Sperm DNA</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Formamide hybridisation buffer:</th>
<th>50% (v/v) Formamide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5X SSPE</td>
</tr>
<tr>
<td></td>
<td>5X Denhardt's solution</td>
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<td>1% (w/v) SDS</td>
</tr>
<tr>
<td></td>
<td>200 μg/ml Salmon Sperm DNA</td>
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</table>

<table>
<thead>
<tr>
<th>50X Denhardt's solution:</th>
<th>1% (w/v) BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% (w/v) Ficoll 400</td>
</tr>
<tr>
<td></td>
<td>1% (w/v) Polyvinylpyridine (PVP)</td>
</tr>
</tbody>
</table>

2.3.3.3.1 *Membrane Stripping for Reprobing*

For successful removal of probes, membranes were never allowed to dry during or after hybridisation and washing. To strip, a boiling solution of 0.1% (w/v) SDS was poured onto the nylon membrane and allowed to cool to room temperature on a shaking platform. This was repeated twice. The membrane briefly rinsed with distilled water to remove the stripping solution, blotted dry and either re-probed with another end-labelled oligonucleotide as above or stored between Saran wrap for future use.
2.3.3.4 Microarray Analysis

This analysis was performed in collaboration with our colleague David Barrass who optimised the conditions and perfected this method for its applications to examine splicing in yeast.

2.3.3.4.1 Microarray slides

Microarray slides were printed at SCGTI University of Edinburgh. They contain oligonucleotide probes corresponding to intron-containing genes of the yeast genome. Each gene has four probes: one probe is to detect the 3’ exon, one to the 5’ splice site, one to the mature junction formed on splicing and one to intron itself.

There are some controls: probes against yeast intronless genes and some to snRNAs; all of these found within introns and some produced by RNA polymerase II. Each probe has 35 – 51 bases (average 41 bases), with a melting temperature of 80-90°C. The probes were printed in triplicate onto the surface of the poly-lysine coated slides where they bind non-covalently. Labelled target cDNA (derived from yeast total RNA) hybridizes to these probes specifically.

2.3.3.4.2 Target labelling

Labelling the target was done as follows: total yeast RNA (section) was prepared from wild-type and temperature sensitive cell cultures matched in pairs having similar culture density. Between 20–50 µg of RNA was mixed with 9 µl of gene specific primer mix (MWG) and the volume adjusted to 19 µl with DEPC-treated water. The specific primer mix consists of oligonucleotide primers (MWG) of 20 – 24 bp that hybridises to the RNA species recognised by the microarray probes. They are designed to amplify an anti-sense target for each of the transcripts represented on the array.

The RNA solution was denaturated at 70°C for 10 minutes and then placed on ice. To this, an equal volume of labelling mix containing the reverse transcriptase and Cy3 / Cy5 labelled deoxynucleotides was added. The labelling reaction was
incubated in the thermoblock (PTC-100 Hot Lid reactor (MJ Research)) overnight at 47°C.

Labelling mix:  
1X cDNA synthesis buffer  
0.2 M DTT  
7.5 mM of each dATP, dCTP, dGTP and 3.5 mM dTTP  
0.01 M Cy3/C5 labelled dTTP (Amersham Pharmacia)  
20 U RNase inhibitors (Invitrogen)  
30 U Thermoscript reverse transcriptase (Invitrogen)

5X cDNA synthesis buffer:  
250 mM Tris-acetate, pH 8.4  
375 mM Potassium Acetate  
40 mM Magnesium Acetate

2.3.3.4.3 Target purification

After this incubation, the labelled cDNA (microarray target) was purified by adding RNA hydrolysis solution, followed by incubation at 65°C for 30 minutes and the neutralisation with Tris-HCl, pH 7.5 at 4°C for at least 5 minutes. Following this, Cy3 and Cy5 targets were combined (in order to form part of one hybridisation) and mix with 20 µg of human Cot1 DNA (Invitrogen). The mixture was applied to Microcon YM-100 purification column (Millipore) and spun to filter-purify the target with two washings of the column.

RNA hydrolysis solution:  
0.5 M NaOH  
250 mM EDTA, pH 8.0
2.3.3.4.4 Pre-treatment and hybridisation

Micro-array slides were treated in blocking solution for 15-20 minutes before being washed. Then they were incubated in pre-hybridisation solution for 45 minutes at 46°C. The incubation step was followed by rinsing in water, dehydration in 95% ethanol and drying by spinning for 1 minute at 1000 rpm (MSE Mistral 1000 centrifuge).

The labelled target was added to slides, covered with cover-slips and the slides were tightly sealed in humidified hybridisation chambers. The hybridisation was performed overnight at 46°C.

After hybridisation, the slides were washed for five minutes each in three washing solutions: wash solution 1 (1X SSC, 0.2% SDS), wash solution 2 (0.1X SSC, 0.2% SDS) and wash solution 3 (0.1X SSC). Finally the slides were rinsed in water and dried as before.

Blocking solution: 92% (v/v) 1-methyl-2-pyrilidinone (NMP)
8% (v/v) Boric acid, pH 7.0
1.63% (w/v) Succinic Anhydride

Pre-hybridisation solution: 5X SSC (Table 2.4)
0.1% SDS
1% (w/v) BSA

2.3.3.4.5 Scanning and data analysis

Arrays were scanned with a scanner Affymetrix 428. The conversion of fluorescence signals to numerical values and scatter plots were performed using Array ProAnalyser software.
2.3.3.5 *In vitro* Transcription of Actin pre-mRNA

2.3.3.5.1 Preparation of DNA template

Plasmid p283 containing the actin gene (Table 2.8) was used for T7 polymerase - directed *in vitro* transcription reaction. The plasmid (500ng) was linearized with *Bam*HI restriction enzyme in a total volume of 20 µl as in section 2.3.2.4. Following digestion, 1 µl of the template DNA was directly used in *in vitro* transcription reaction.

2.3.3.5.2 Transcription Reaction

The transcription reaction was set up in a clean Eppendorf and incubated at 37°C for 30 minutes.

The reaction mix was as follows:

- Liniarised template DNA: 1 µl
- T7 buffer: 1.5 µl
- 10 mM CTP, GTP, UTP: 1 µl
- 400 µM ATP: 1 µl
- \( [\alpha-^{32}\text{P}] \) ATP (~3000 Ci/mmol): 1 µl
- sterile, distilled water: 11.7 µl
- RNasin: 0.3 µl
- T7 RNA polymerase: 0.5 µl

T7 buffer: 0.4 M Tris-HCl, pH 8.0
- 0.1 M MgCl₂
- 0.2 M DTT
- 0.1 M NaCl
2.3.3.5.3 Purification of transcript

The actin transcription reaction volume was increased to 100 µl with DEPC-treated water and purified from proteins, unwanted nucleotides and salts by phenol: chloroform:isoamyl alcohol (25:24:1) extraction (section 2.3.1.1) and ethanol precipitation (section 2.3.1.2). The RNA pellet was dried under vacuum and resuspended in 50 µl of DEPC-treated water. The transcript could be stored at -20°C for up to 5 days.

2.3.3.6 In vitro splicing

In order to analyse if a whole yeast cell extract (see section 2.4.3) is able to splice (i.e. formation of spliced mRNA and splicing intermediates), in vitro splicing was performed using radiolabelled actin pre-mRNA transcript (section 2.3.3.5) as a substrate.

The following reaction mix was set up in a sterile Eppendorf:

- 10x splicing buffer: 1 µl
- 30% (w/v) PEG -8000: 1 µl
- DEPC-treated water: 2.5 µl
- Splicing extract: 5 µl
- In vitro actin pre-mRNA (~1000cps): 0.5 µl

The reaction was incubated at 24°C for 25 minutes and then terminated by placing on ice. Two microlitres of Proteinase K solution was added, and the contents were gently mixed and incubated at 37°C for 45 minutes. Following this incubation, 200 µl of splicing cocktail was added and the resulting mixture extracted twice with 200 µl of phenol:chloroform:isoamyl alcohol 25:24:1 (section 2.3.1.1) to remove the protein debris. The supernatant containing the RNA was precipitated by the addition of 600 µl of ethanol (abs.) (section 2.3.1.2) and centrifuged at 14,000 rpm for 20 minutes at 4°C. The RNA pellet was washed in 70% (v/v) ethanol and dried under vacuum. Ten microlitres of Sanger loading dye (section 2.3.3.2) were added and the
samples boiled for 5 minutes. The prepared RNA was loaded on a 7% denaturing polyacrylamide gel (section 2.3.3.2) and run in 1X TBE buffer (Table 2.4) at 24 W for 1 hour. The gel was vacuum-dried for 2 hours at 75°C and exposed to autoradiography film at -70°C.

10x splicing buffer:  
600 mM KPO$_4$, pH 7.5  
25 mM MgCl$_2$  
20 mM ATP

Proteinase K solution:  
1 mg/ml Proteinase K  
50 mM EDTA, pH 8.0  
1% (w/v) SDS

Splicing cocktail:  
50 mM NaoAc, pH 5.3  
1 mM EDTA, pH 8.0  
0.1% (w/v) SDS  
25 μg/ml *E. coli* tRNA

2.3.3.7 Native gel electrophoresis

Native gel electrophoresis was carried out using an adaptation of the method of Pikielny *et al.* (1986). This system separates the three splicing complexes: pre-spliceosome (complex III or A), inactive spliceosome (complex I or B) and active spliceosome able to support the two splicing steps (complex II or C).

Two 16 X 16 cm glass plates separated by 1.5 mm spacers were tapped together and pre-warmed at 65°C. To seal the plates, a 3-4 cm blocking gel was poured between the plates and the gel-assembly left to set at 65°C for 5 minutes. A separating gel was then poured on the top of the blocking gel up to the top of the plates and a 10 X 10 cm comb was inserted. The gel was allowed to polymerise at room temperature for 3-4 hours and then stored at 4°C overnight covered in Saran wrap. Non-denaturing gels were pre-run at 4°C for 1 hour in pre-chilled 1X Native
gel running buffer (80V).

Splicing reactions were set up as in section 2.3.3.6 and terminated on ice. An equal volume of ice-cold Q buffer was added to each reaction and then incubated for 10 minutes on ice. Five microlitres of ice-cold RNP loading dye were added to each sample then loaded on the gel. The gel was run at 4°C overnight at 30V until the xylene blue dye front had reached the blocking gel. Once the electrophoresis terminated, the gel was covered in Saran wrap and exposed to autoradiography film at -70°C.

**Blocking gel:**

1 ml 29.2% (w/v) acrylamide : 0.8% (w/v) bis-acrylamide
100 µl 0.5M EDTA
250 µl 10X TB
3.65 ml DEPC-treated water
50 µl 10% ammonium persulphate (APS)
20 µl TEMED

**Resolving gel:** 0.25 g agarose was melted in 40 ml DEPC-treated water and then the mixture incubated to 65°C until use. Five milliliters of acrylamide [29.2% (w/v) acrylamide : 0.8% (w/v) bis-acrylamide] were mixed with 2.5 ml 10X TB buffer, 1 ml 0.5 M EDTA and 2.5 ml glycerol and incubated at 65°C. The agarose mixture was added to the acrylamide mixture, the total volume was adjusted to 50 ml with pre-warmed DEPC-treated water (65°C). Four hundred microlitres of 10% ammonium persulphate (APS) and 50 µl of TEMED were added and the gel was poured immediately.
<table>
<thead>
<tr>
<th>Buffer Type</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>4X Q mix</strong></td>
<td>1.6 M KCl</td>
</tr>
<tr>
<td></td>
<td>8 mM Magnesium Acetate</td>
</tr>
<tr>
<td></td>
<td>80 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>64 mM Tris-HCl, pH 7.5</td>
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<tr>
<td><strong>Q buffer</strong></td>
<td>1X Q mix</td>
</tr>
<tr>
<td></td>
<td>2.8 μg/μl yeast total RNA</td>
</tr>
<tr>
<td><strong>RNP Loading dye</strong></td>
<td>50% Ficoll</td>
</tr>
<tr>
<td></td>
<td>2.5X TB</td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.2% Bromophenol blue</td>
</tr>
<tr>
<td></td>
<td>0.4% Xylene cyanol</td>
</tr>
<tr>
<td><strong>10X TB</strong></td>
<td>1.2 M Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>400 mM Boric acid</td>
</tr>
<tr>
<td><strong>10X Native gel running buffer</strong></td>
<td>5X TB</td>
</tr>
<tr>
<td></td>
<td>100 mM EDTA, pH 8.0</td>
</tr>
</tbody>
</table>
2.4 Protein methods

2.4.1 Spectrophotometric Quantification of Proteins

Protein concentrations were determined by the Bradford method using the Bio-Rad Protein Assay and following the manufacturer’s instructions. Briefly, a series of BSA standards were prepared by diluting 10 mg/ml BSA (NEB) in sterile, distilled water to give four to five dilutions between 0.2 and 0.9 mg/ml. One hundred microliters of each standard solution was mixed with 5 ml diluted (1:5) Bradford Dye Reagent (Bio-Rad), vortexed and allowed to stand for 5 minutes at room temperature. The OD$_{595}$ of each sample was measured (with a 0 mg/ml solution as a blanc) and the values used to plot a graph of BSA concentration versus OD$_{595}$. The protein sample of unknown concentration was simultaneously treated in the same manner and the concentration was extrapolated from the standard plot. Each protein concentration was assayed in triplicate.

2.4.2 Crude Extraction of Total Cellular Protein from Yeast

Ten milliliters of the appropriate medium (section Table 2.2) was inoculated with a single yeast colony and incubated at the required temperature overnight. The following day the OD$_{600}$ was measured and a total of 3 OD$_{600}$ units of cells were harvested by centrifugation for 5 min at 3500 rpm (Mistral 1000 centrifuge). The pellet was resuspended in 500 $\mu$l of 0.2 M NaOH, transferred to a clean Eppendorf and left on ice for 10 minutes. Fifty microlitres of 50% Tri-sodium acetate (TCA) were added and the precipitate incubated on ice for at least 10 minutes. The suspension was centrifugated for 5 minutes at 14,000 rpm and the supernatant disposed. The pellet was resuspended in 35 $\mu$l of dissociation buffer and then 15 $\mu$l of 1M Tris base were added to neutralise the TCA. The extract was stored at -20°C.
until use. For SDS-PAGE followed by Western blotting, 5-8 µl of extract were heated to 95°C for 10 minutes prior to loading on a polyacrylamide gel (see section 2.4.4).

Dissociation buffer: 0.1 M Tris-Cl, pH 6.8
4 mM EDTA, pH 8
4% SDS
20% Glycerol
2% β-mercaptoethanol
0.25% Bromophenol blue

2.4.3 Large Scale Extraction of Yeast Total Cell Protein (Splicing Extract)

One hundred milliliters of the appropriate medium (section Table 2.2) was inoculated with a single yeast colony and incubated overnight at the required temperature. This culture was used to inoculate 3-4 litres of medium to an OD_600 of 0.1 and the cells were grown further until the OD_600 reached 0.5 - 1.0. The cells were harvested by centrifugation at 5000 rpm for 5 minutes (Beckman, JLA 10.5000 rotor) and the pellet was resuspended in 50 ml of AGK buffer. Cells were sedimented at 3500 rpm for 5 minutes (Mistral 1000 centrifuge), resuspended in 20 ml of AGK buffer and centrifuged once again as above. The supernatant was discarded, the pellet was weighted and resuspended in 400 µl of AGK buffer for each gram of cell pellet. To this suspension, DTT concentration was adjusted to 2 mM and then 10 µl of 100X protease inhibitors (Sigma) was added. Using a sterile syringe or a 1ml Gilson pipette, the cell suspension was transferred drop-wise in a 50ml Falcon tube full of liquid nitrogen. This cell pellet could be either processed further immediately or stored at -70°C for at least one year with no loss of activity.

Using a mortar containing liquid nitrogen the frozen cell pellet was ground to a fine powder with occasional addition of liquid nitrogen to ensure that the pellet did
not thaw. This frozen powder was then transferred to a chilled polycarbonate centrifuge tube and centrifuged at 17,000 rpm for 30 minutes at 4°C (Beckman JA 25.50 rotor, pre-chilled to 4°C). Without disturbing the lipid layer, the supernatant was transferred to a chilled Ultra plus polycarbonate tube (Nalgene) and centrifuged at 40,000 rpm for 1 hour at 4°C (Beckman 70.1 Ti rotor, pre-cooled to 4°C). The final supernatant was transferred to a dialysis membrane (10 kDa cut-off) and dialysed against two changes of 1.5 litres of ice-cold Buffer D at 4°C, for 3 hours. The splicing extract was then placed in pre-chilled Eppendorfs and centrifuged for 10 minutes at 4°C. The resulting supernate was aliquoted into fresh pre-chilled microfuge tubes and stored at -70°C. The splicing extract was thawed slowly on ice prior to use.

AGK buffer: 10 mM HEPES, pH 7.9
1.5 mM MgCl₂
200 mM KCl
10% (v/v) Glycerol

Buffer D: 20 mM HEPES, pH 7.9
50 mM KCl
0.2 mM EDTA, pH 8.0
20% (v/v) Glycerol
0.4 mM DTT (added immediately before use)
1 tablet of Protease Inhibitors (Roche) per 1.5 L of buffer D

2.4.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.4.4.1 Novex Mini Gels

Protein samples to be analysed by SDS-PAGE were, unless otherwise stated, mixed with an equal volume of 2X SDS loading buffer, heated for 5-10 minutes at 70°C and centrifuged at 14,000rpm for 20 seconds. The samples were loaded onto a pre-cast 4-
12% polyacrylamide gel (Novagen), alongside a molecular weight protein marker of known size (SeeBlue Plus2 Pre-Stained Protein Standard, Invitrogen) for reference. The gels were run at 200V for 50 minutes, in a Novex Mini-cell (Novagen) with 1X MOPS (Table 2.4) as buffer.

2X SDS Loading buffer: 100 mM Tris-HCl, pH 6.8
200 mM DTT (added immediately before use)
4% (w/v) SDS
0.2% (w/v) Bromophenol blue
20% Glycerol

2.4.4.2 Classic Gels

In order to get a better resolution of the protein bands, the SDS-PAGE was performed using the classic gel system. The SDS polyacrylamide gel was poured between two taped 18 x 20 cm glass plates separated by 1 mm spacers. The resolving gel solution was prepared, poured between the plates and overlaid with isopropanol. The gel was then left to set at room temperature, the isopropanol was washed off with sterile, distilled water and the stacking gel was poured and an appropriate comb inserted to create the necessary loading wells. After polymerisation was complete, the sealing tape was removed from the plates and the comb was gently taken out. The plates were fixed into the electrophoresis apparatus (Cambridge Electrophoresis) and the chambers were filled with 1X Protein Running Buffer. Protein samples were treated as previously and loaded onto the gel along with the molecular weight protein marker (SeeBlue Plus2 Pre-Stained Protein Standard, Invitrogen). The gel was run at 180V for 7 hours.
10% resolving gel solution: 12 ml 30% (w/v) acrylamide
2.34 ml 2% (w/v) bis-acrylamide
9 ml 1.5M Tris-Cl, pH 8.8
360 µl 10% (w/v) SDS
12.3 ml sterile, distilled water
360 µl 10% (w/v) ammonium persulfate
36 µl TEMED

Stacking gel:
1.7 ml 30% (w/v) acrylamide
650 µl 2% (w/v) bis-acrylamide
1.250 ml 1M Tris-Cl, pH 6.8
6.4 ml sterile, distilled water
100 µl 10% (w/v) ammonium persulfate
10 µl TEMED

10X Protein Running Buffer: 250 mM Tris Base
1.9M Glycine
1% (w/v) SDS

2.4.5 Western Blotting

2.4.5.1 Electrophoretic Transfer of Proteins to PVDF Membrane

Proteins were transferred electrophoretically from SDS polyacrylamide gel to Hybond-P membrane (Amersham Pharmacia) using the Bio-Rad transfer system and following the manufacturers’ protocol. The transfer was performed in 1X Western Transfer Buffer at 100V for 2 hours or at 20V over-night. Completion of transfer was assessed by the pre-stained marker.

10X Western Transfer Buffer: 1.5 M Glycine
200 mM Tris-HCl, pH 8.3
2.4.5.2 Antibody Binding

Non-specific protein interactions were blocked by incubating the membrane in 25 ml of fresh Blocking Buffer for 1 hour at room temperature, with constant shaking. The membrane was washed three times with 25 ml of 1X TBS-T buffer (Table 2.4) before being incubated in 10 ml Blocking Buffer* with the appropriate dilution of primary antibody (usually 1:1000). This incubation was performed overnight, at 4°C, with gentle agitation. The following day the membrane was washed four times in 50 ml TBS-T buffer, each time for 10 minutes. The HRP-conjugated secondary antibody in 10 ml of Blocking Buffer (1:1000 dilution) was added and the incubation continued for 1 hour at room temperature, with gentle shaking. Finally, the membrane was washed for four times for 10 minutes each with 50 ml of 1X TBS-T buffer.

Blocking Buffer: 1X TBS-T buffer
5% (w/v) non-fat dry milk

*For the polyclonal antibodies, the non-fat dry milk in the Blocking Buffer was substituted with Bovine Serum Albumin (BSA).

2.4.5.3 Enhanced Chemiluminescence (ECL)

Two millilitres of developer solution (Amersham) was prepared as described by the manufacturer, applied to the membrane and incubated for 1 minute at room temperature with gentle agitation. The membrane was drained of excess developing solution, wrapped in Saran wrap and exposed to X-ray film. Exposure times were varied depending on the strength of the signal.
2.4.6 *In vitro* Transcription/Translation of Proteins

*In vitro* coupled transcription/translation systems (TNT systems) are cell-free protein synthesizing tools for the *in vitro* expression of proteins from cloned genes. Briefly, a DNA template is introduced in a rabbit reticulocyte lysate (as used in the present work) that contains the cellular components necessary for gene transcription and protein synthesis: RNA polymerases, transcription factors, tRNA, ribosomes, amino acids, and initiations, elongation and termination factors. The system also contains a variety of post-translational processing activities including acetylation, proteolysis and some phosphorylation.

2.4.6.1 Preparation of DNA template

The gene or gene fragment needed to be expressed was amplified by PCR (section 0) using oligonucleotide primers designed as follows: the 5' oligonucleotide primer, F, contained a T7 phage polymerase promoter required for transcription initiation from the DNA template (Figure 2.3). In this work to produce Prp45p, full length and truncated versions, *PRP45*-containing plasmid pRS-45 was used. When amplifying from an internal AUG (to produce internal regions of the protein of interest), a Kozak consensus sequence (CCACCATG) was also introduced in 5' primer to ensure efficient translation initiation. The 3' primer, R, should contain an in-frame termination codon (usually, TAA) if the native termination codon was not present. Following amplification of the desired sequence, the resulting DNA template was analysed by agarose gel electrophoresis (section 2.3.2.8) to ensure that the correct product had been amplified. Then PCR-generated DNA fragments were used directly in the TNT reaction without purification.
2.4.6.2 *In vitro* coupled transcription-translation reaction

Between 0.2 – 2 µg of DNA template were added to the components of Rabbit Reticulocyte TNT kit (TNT T7 Quick for PCR DNA, Promega) according to the manufacturer’s instructions and incubated at 30°C for 90 minutes. L-[³⁵S]Methionine (~1000 Ci/mmol) was included in the reaction in order to be incorporated in the *in vitro* translated protein and thus, to allow the protein detection by autoradiography (Figure 2.3).

Following incubation, 5 µl of each reaction was mixed with 2X SDS Loading Buffer (section 2.4.4), heated at 70°C for 5 minutes and the samples were analysed by SDS-PAGE (section 2.4.4). The gel was then covered in Saran wrap, dried on a Gel-Vac vacuum gel drier (Hybaid) for 2 hours at 75°C. Then the gel was subjected to autoradiography by exposing to BioMax MR film (Kodak) at room temperature.
2.4.7 Co-immunoprecipitation of Proteins from Yeast Extract

To show protein-protein interaction *in vitro*, co-immunoprecipitation assay was performed.

Twenty-five microliters of HA<sub>2</sub>-tagged *PRP46* splicing extract prepared as in section 2.4.3 was depleted of ATP (and thus of splicing activity) by adding 2 mM glucose and incubating at 24°C for 20 minutes. Five microliters of <sup>35</sup>S-labelled polypeptides produced by TNT reaction (section 2.4.6) and 4 µl of anti-HA antibodies (12CA5, Roche, Table 2.10) were added and the total NaCl concentration was adjusted to 150 mM with IP<sub>300</sub> Buffer. The reaction was incubated on ice for 1 hour. Protein A-Sepharose (PAS) beads (10 mg/sample) were swollen in 1 ml of NTN Buffer for 30 minutes, washed twice in 1 ml NTN Buffer and twice with ice-cold IP<sub>150</sub> Buffer before being suspended in 265 µl of ice-cold IP<sub>150</sub> Buffer per sample. PAS beads were then added to each sample and incubated on a rotating wheel for 2 hours at 4°C. Five microlitres of each supernate (1.6%) were then subtracted and used as <sup>35</sup>S-labelled protein controls. The remaining suspension was washed three times with 1 ml of chilled IP<sub>150</sub> Buffer and the pellets were finally resuspended in 25 µl of 2X SDS loading buffer (section 2.4.4). Samples were then heated at 70°C for 10 minutes, centrifuged at 1,000 rpm for 60 seconds and subjected to SDS-PAGE (section 2.4.4). The control supernates were mixed with an equal volume of 2X SDS loading buffer, heated as above and loaded alongside the immunoprecipitation samples. In order to visualise any co-immunoprecipitated [<sup>35</sup>S]-Methionine-labelled polypeptides, the gel was covered in Saran wrap and vacuum-dried as in section 2.4.6.2. Then this was subjected to autobiography with BioMax MR film (Kodak) at room temperature.

**NTN Buffer:**

- 150 mM NaCl
- 50 mM Tris-HCl, pH 7.5
- 0.1% (v/v) Nonidet P40
**Chapter 2: Materials and Methods**

**IP\textsubscript{300} Buffer:**
- 12 mM HEPES, pH 7.9
- 300 mM NaCl
- 5 mM MgCl\textsubscript{2}
- 0.1% (v/v) Nonidet P40

**IP\textsubscript{150} Buffer:**
- 6 mM HEPES, pH 7.9
- 150 mM NaCl
- 2.5 mM MgCl\textsubscript{2}
- 0.05% (v/v) Nonidet P40

### 2.4.8 Phosphorylation Co-Immunoprecipitation

In order to assay if the protein of interest (Prp45p in the present work) is post-translationally modified by phosphorylation, a co-immunoprecipitation assay was performed followed by λ-Protein Phosphatase Digestion.

Fifty microliters of 13-Myc-tagged *PRP45* splicing extract prepared as in section 2.4.3 was resuspended in 250 µl of Phosphatase and Protease Inhibitors (PPI) Buffer. The mixture was added to 40 µl of agarose beads coupled with mouse monoclonal anti-c-Myc antibodies (Santa Cruz Biotechnology, Table 2.10). Prp45p-Myc was immunoprecipitated for 2 hours at 4°C on a rotating wheel and 50 µl of the supernatant was taken off and used as a protein loading control. The immunoprecipitate was washed three times with PPI Buffer, twice with 800µl of 1X PBS Buffer (Table 2.4) and then twice with 500µl of LPP reaction buffer. Half of the agarose beads were resuspended in 50 µl of LPP buffer and 400 U of λ-Protein Phosphatase (New England Biolabs) were added. The Phosphatase digestion reaction was incubated for 45 minutes at 30°C. The agarose beads were then spun down and heated in 20 µl of Dissociation buffer (section 2.4.2). The samples were separated on 10% poly-acrylamide gel as described in section 2.4.4.2 and Prp45p was analysing by immunobloting with rabbit polyclonal anti-c-Myc antibodies (Table 2.10).
PPI Buffer: 50 mM Hepes, pH 7.9
75 M KCl
1 mM EGTA
1 mM MgCl₂
0.1% (v/v) NP40
1 mM Sodium Vanadate
50 mM Sodium Fluoride
½ tablet Protease Inhibitors per 10 ml of buffer

LPP Buffer: 1X λ-Protein Phosphatase Buffer
2 mM MnCl₂
Chapter 3  Prp45p and the Spliceosome
3.1 Introduction

The two–hybrid screens performed previously (Albers et al., 2003) not only detected a mutual interaction between Prp45p and Prp46p but also mapped the regions of contact within each protein. Thus, Prp45p through a portion of the conserved SNW domain (amino acids 54–204) interacts with the Prp46p cluster of seven WD-repeat units (amino acids 127–432). However, this kind of analysis does not ascertain if the interaction between the bait protein and the prey protein leading to the transactivation of the reporter gene is direct or if some other factor(s) contributes to it. So, to confirm a two-hybrid interaction, co-immunoprecipitation experiment must be employed and this is described in this chapter. The data obtained showed the importance of yeast N-terminal domain of Prp45p, which might confer extra functions to the yeast protein that are not complemented in a Prp45p-depleted strain by the human homologue, SKIP.

Albers et al. (2003) found Prp45p associated with the spliceosome before the first step of splicing but the exact timing of this association was not established. Therefore it was of interest to determine this by resolving spliceosome complexes that form in yeast splicing extract depleted of Prp45p.

Throughout this work monitoring of \textit{PRP45} expression has been achieved using a \textit{P}_{GAL} regulated \textit{PRP45} strain named YMA45/2. This haploid yeast strain (Albers et al., 2003) has part of the \textit{PRP45} promoter substituted with a cassette containing: the \textit{HIS3} gene marker, the yeast \textit{Gal1-10} promoter region (\textit{P}_{GAL}), and \textit{S. aureus} Protein A epitope (see Table 2.6 and Figure 4.1). The presence \textit{P}_{GAL} allows the expression of \textit{PRP45} to be turned on by growing the strain with galactose as sole carbon source and turned off in the presence of glucose in the growth medium.
3.2 Prp45p and Prp46p interact in vitro

In order to validate the two-hybrid interaction between Prp45p and Prp46p, co-immunoprecipitation experiments were employed using yeast extract containing HA2-tagged Prp46p and in vitro translated Prp45p, full length or deletion derivatives, produced with TNT transcription/translation kit (Promega). The template DNA provided by PRP45–containing plasmid pRS-45 (see Table 2.9 and section 4.2.1) was amplified by PCR according to the protocol described in section 2.3.2.6. The primers were designed to produce the following peptides: WT representing full-length Prp45p; R46 (amino acids 54-218), R1 (amino acids 1-218), R2 (54-291) and R4 (54-379) all include the minimum region of interaction with Prp46p found in two-hybrid screen (amino acids 54-204); R5 (218-379) is the C-terminal region of Prp45p and constitutes the negative control for the co-immunoprecipitation (Figure 3.1.A). The PCR products were transcribed using T7 polymerase and translated into the corresponding peptides in the presence of 35S-methionine (see section 2.4.6) so that, after resolving by SDS-PAGE, they could be visualised by autoradiography (Figure 3.1.B).

Following the TNT reaction, some of the peptides (WT, R2, R4) were resolved as multiple bands, a common problem with this system (Figure 3.1.B). The band corresponding to the correct peptide was the slowest migrating one while the others were caused by the incorrect initiation of translation at ATG codon downstream of the first one. Some of these fast migrating bands could also be breakdown products of the original peptide.

Another feature of the synthesized peptides was their slower electrophoretic mobility that corresponded to a 5-8 kDa increase in their molecular weight. This migration behaviour was caused by the highly charged nature of Prp45p and is discussed in detail in Chapter 5.
Figure 3.1 Schematic diagram of different truncated forms of Prp45p synthesized *in vitro* by transcription and translation.

A. Wild-type Prp45p (WT) is represented as a box and the conserved SNWKN as a blue bar. The Prp45p truncated derivatives (R1 - R5) are depicted below as black bars. R-M and R-H depicted as red bars are the minimum regions of interaction with Prp46p found in two-hybrid screens performed in our lab and at Hybrigenics, respectively. The numbers represent amino acid positions.

B. The peptides depicted in A. were produced *in vitro* using Promega TNT T7 Quick for PCR DNA. Five microliters of the TNT reactions were mixed with an equal volume of 2X SDS Loading Buffer and loaded onto 4-12% PAA gel (Novex Minigel). The gel was dried and $^{35}$S-labelled peptides were visualised by autoradiography. As a positive control, luciferase (Luc) was produced using the same protocol, while the negative control (−DNA) was the reaction mix incubated without DNA template. The sizes in kDa of the marker proteins are indicated on the left.
Figure 3.2 Analysis of the *in vitro* interaction between Prp46p and Prp45p. Yeast splicing extract containing HA$_2$-Prp46p was ATP-depleted and incubated with monoclonal anti-HA antibodies and *in vitro* produced Prp45 polypeptides (Figure 3.2) for 1 hour on ice. Protein A-sepharose beads were added and the incubation continued for 2 hours at 4°C. As controls, the precipitations were performed without yeast extract. The precipitated proteins (Precipitates), controls and supernates (1.6%) were analysed by SDS-PAGE and the $^{35}$S-labelled polypeptides visualised by autoradiography.
Next, *in vitro* translated peptides were used to check the interaction with Prp46p. As shown in Figure 3.2, wild-type Prp45p and the N-terminal fragment, R1, precipitated with HA2-tagged Prp46p from yeast extract. In contrast, the other peptides R46, R2 and R4, all containing the minimum region of interaction with Prp46p (amino acids 54-204), as identified in two-hybrid screen, failed to precipitate Prp46p. As expected, R5 did not show any association with Prp46p. This behaviour was not a result of peptide degradation, as the peptides were present in the supernates (Figure 3.2).

These data confirmed the two-hybrid screens results of a physical interaction between the two proteins either directly or indirectly. To show a direct interaction between the two proteins experiments were done with His6-tagged Prp46p produced in *E. coli* (A. Diment, this lab; data not shown). Unfortunately they were not successful, due perhaps to some post-translational modification(s) of Prp46p that in contrast to yeast, cannot take place in *E. coli*. However a direct interaction between the two proteins was demonstrated performing the co-immunoprecipitation assay the other way around, with the *in vitro* synthesized Prp46p polypeptides and His6-tagged Prp45p produced in *E. coli* on a pET19b vector (Novagen). These results are also presented in Albers et al., 2003.

### 3.3 Prp45p is involved in formation of the active spliceosome

In order to investigate at what specific stage of spliceosome assembly Prp45p functions, splicing extracts from YMA45/2 cells grown either under permissive conditions (galactose-based medium) or repressive conditions (glucose-based medium) were incubated under *in vitro* splicing conditions and the complexes that formed on pre-mRNA at different times were resolved on native gel electrophoresis (Figure 3.3.B).
Figure 3.3 Pre-mRNA splicing and time course of formation of splicing complexes in Prp45p-depleted cells.

A. Yeast splicing extracts were prepared from YMA45/2 cells grown for 14 hours in permissive, galactose-based liquid medium (YMGRS-sup) (lane 2) and in non-permissive, glucose-based medium (YPDA) (lanes 3-5). The splicing reactions were initiated by the addition of $^{32}$P-labelled $ACTI$ pre-mRNA as described in section 2.3.3.5. Splicing activity of Prp45p-depleted extract could be restored on addition of 0.4 μg of recombinant His$_6$-Prp45p or 1 μl of in vitro translated Prp45p (lane 5). The actin lane (lane 1) contains only the $^{32}$P-labelled actin pre-mRNA. The positions of the $ACTI$ pre-mRNA, lariat intermediate and excised intron are noted on the right.

B. Splicing reactions set up as in A., (as in lanes 2 and 3) incubated for the indicated times, then stopped on ice and incubated in the presence of 1.4 μg/μl of yeast total RNA for 10 minutes before loading onto a 3% polyacrylamide/0.5% agarose gel (see section 2.3.3.7). The positions of the splicing complexes (I, II, III) are indicated on the left. U represents the unassembled pre-mRNA.
A. \textit{GAL1: PRP45}  
\begin{align*}  
\text{actin} & \quad \text{GAL} & \quad \text{GLU} \\
1 & \quad 2 & \quad 3 & \quad 4 & \quad 5 \\
- & \quad + & \quad - & \quad + \\
\text{His}_{5}\text{-Prp45p} & \quad \text{\textsuperscript{35}S-Prp45p} \\
\end{align*}

B. \textit{GAL1: PRP45}  
\begin{align*}  
\text{Time (min)} & \quad 5 & \quad 15 & \quad 25 \\
\text{GAL} & \quad \text{GLU} \\
1 / B & \quad 2 & \quad 3 & \quad 4 & \quad 5 & \quad 6 \\
\text{II / C} & \quad \text{III / A} \\
\text{U} \\
\end{align*}
In parallel it was tested whether Prp45p depleted extract was functional (Figure 3.3.A) by the addition of recombinant His$_6$ tagged-Prp45p produced in E. coli (A. Diment, this lab) (lane 4) or Prp45p synthesized in vitro, in rabbit reticulocytes lysate by TNT reaction (see 2.4.6 and section 3.2) (lane 5). As shown previously (M. Albers, PhD Thesis), splicing extract prepared from YMA45/2 cells grown under non-permissive conditions (GLU) could not support splicing in vitro (lane 3) but extract prepared from the same strain grown under permissive conditions (GAL) could process actin pre-mRNA (lane 2). The splicing defect was partially complemented in vitro by the addition of recombinant Prp45p (0.4 μg) or radiolabelled Prp45p (1 μl), showing that Prp45p was the only factor required to make this splicing extract functional and that could be used for further analyses. A range of different concentrations of Prp45 protein (0.2, 0.8, 1.0, 1.2 μg of recombinant protein or 0.5, 2.0, 2.5, 3.0 μl of in vitro translated protein) was used to reconstitute the loss of splicing activity but the results did not show a better complementation (data not shown).

The observed defect of Prp45p-depleted cells was associated with a specific defect in spliceosome assembly (Figure 3.3.B). Splicing reactions run on native gel were separated as described previously (Pikielny et al., 1986) into three discrete complexes named I, II and III (see section 2.3.3.7). As expected, pre-mRNA from the Prp45p-complete extract was rapidly assembled into the prespliceosome (complex III or A) that was converted into the to inactive spliceosome (complex I or B) and then to the final active spliceosome, complex II or C, that contains the intermediates and products of the splicing reaction (lane 1-3). In contrast, extracts prepared from glucose-repressed $P_{GAL}$: PRP45 cultures failed to support the formation of active spliceosome while complexes III and I assembled but at a slow rate (lanes 4-6).

In conclusion, it can be assumed that the absence of Prp45p did not significantly affect earlier steps in spliceosome assembly but was necessary for the formation of catalytically active spliceosomes.
3.4 Complementation of yeast Prp45p with the human SKIP/Snw1p fragments

The human homologue of yeast Prp45p (Snw1p, SKIP or NCoA-62) was previously shown to substitute for the function of Prp45p in yeast cells depleted of this protein (M. Albers, PhD Thesis). Full-length Prp45p presents a 22.8% amino acid identity with full-length SKIP and the sequence homology within the SNW domains of these two proteins reaches 35.5%. Although there are studies revealing the presence of SKIP in spliceosome (Neubauer et al., 1998, Makarov et al., 2002) a direct role of SKIP in pre-mRNA splicing has not been proved until recently (Zhang et al., 2003).

Moreover, some conserved domains found in most of the SNW family members are required for different functions within the cell such as transcription, protein-protein interaction and signalling pathways (Zhang et al., 2001) (Figure 1.4.A).

In order to find out which domain of SKIP is responsible for its ability to substitute for its yeast homologue, and thus the domain required for splicing, the complementation of the growth defect of the Prp45p-depleted strain YMA45/2 was attempted using four SKIP deletion constructs in pGBT9 (kindly gift of J. Figueroa). These plasmids (depicted in Figure 3.4.A) encode for the DNA-binding domain of GAL4 (GBD) fused with the following SKIP domains: 1-200 (corresponding to the prolyl-peptidase (PPIase) binding domain in S. pombe and D. discoideum); 1-300 (containing the proline-rich domain as well as SNW domain with the conserved SNWKN motif); 201-537 (containing all the conserved regions of SNW family proteins: the proline–rich domain, the SNW domain, the SH2-like domain, the C-terminal domain); 334-537 (containing SH2-like domain as well as the conserved C-terminal domain found in all SNW members except S. cerevisiae and P. falciparum) (Figure 3.4.A).

The strain YMA45/2 was transformed with these plasmids as well as with pGBT/SKIP and pMA45 (bearing the LexA-domain fused with PRP45 to detect the efficiency of complementation). As controls, plasmids pGBT9 (producing GBD domain) and pBTM116 (producing LexA domain) were tested in parallel. The transformants were grown on GAL-HW medium for 5 days.
Figure 3.4 Complementation of Prp45p depletion by the human SKIP protein. A. Proposed homology between human SKIP and yeast Prp45p and cartoons of SKIP truncated proteins used for complementation assay. Both proteins are depicted as boxes and the dashed and colored boxes indicate the conserved motifs/domains/common regions among the SNW members. The numbers indicate the amino acid positions.

B. Yeast strain YMA45/2 was transformed with either pGBT9 (producing the Gal4p DNA-binding domain, GBDp); pGBT9-derived fusion plasmids producing the fusion proteins: GBD:SKIP(1-200), GBD:SKIP(1-300), GBD:SKIP(201-536), GBD:SKIP(334-536); LAp (producing LexA protein); pGBT9/SKIP (producing the human SKIP, N-terminally fused to GBDp, GBD:SKIP) and pMA45 (producing Prp45p fused to the LexA protein, LA:Prp45p). Transformants were selected on galactose-based medium (GAL-HW), suspended in serial dilutions in microtiterplates and then transferred onto either galactose (YPGalA-HW) or glucose (YMM-HW) based medium. The plates were incubated for 5 days at 30°C before being photographed.
It can be seen that, under non-permissive conditions (glucose-based medium) when Prp45p was depleted from the cells, none of the SKIP deletion constructs overcame the growth defect. Full-length SKIP complemented the growth defect but not as efficiently as the yeast Prp45p (Figure 3.4).

It can be concluded that, although SKIP represents the functional homologue of Prp45p, none of its highly conserved domains are individually sufficient for yeast Prp45p function. One possible explanation could be the lack of the conserved proline-rich domain and the conserved C-terminal domain found in SKIP and other SNW proteins. These regions might be necessary for splicing in higher eukaryotes and redundant in yeast where their activity may be compensated by other factors. Another explanation might be given by N-terminal domain of yeast Prp45p, distinctive from its counterparts in respect of length (yeast Prp45p is much shorter) and structure, aspects that could provide extra features in folding and structural integrity.

3.5 Discussion

In recent years the existence of protein complexes has been suggested by different approaches, either genetic approaches, like two-hybrid screens, synthetic lethal screens and suppressor screens or biochemical analyses like immunoaffinity purification followed by mass spectrometry. These analyses proved to be successful in identifying protein complexes involved in splicing. It is noteworthy for the present work to mention the Cwc-multiprotein complex identified in budding yeast (Ohi et al., 2002) and its homologue in fission yeast, Cwf-complex (Ohi et al., 2002), the yeast penta-snRNP complex containing the five snRNAs and around 60 proteins (Stevens et al., 2002) or the mammalian 35S U5snRNP complex (Makarov et al., 2002). In all these complexes Prp45p/SKIP and Prp46p/PRLG1 were always present. However, the first indication of an interaction between the two proteins (and others identified in the multiprotein complexes described above) came from the two-hybrid screens performed in our lab (Albers et al., 2003).
The data presented in this chapter confirmed this interaction *in vitro* and specified the Prp45p region of interaction within the N-terminal domain. The interaction requires not only the minimum portion found in two-hybrid screens but also an additional N-terminal end. In addition to this, a similar study (Martinkova *et al.*, 2002) found that the minimum region in Prp45p sufficient for cell survival lies within amino acids 1-190. Neither the N-terminal domain (amino acids 1-119) nor the region within amino acids 53-190 (overlapping with the one found in two-hybrid screens) could complement the loss of the yeast Prp45 protein in Prp45p-depleted cells. In contrast with SNW domain, the N-terminal region (amino acid 1-54) is poorly conserved. It does not contain special known motifs but might promote the correct folding and/or stability of the protein. These data could explain the failure of human SKIP N-terminal derivatives (amino acids 1-200 and 1-300) to complement the loss of its yeast homologue (Figure 3.4). The importance of this domain is reflected by the temperature-sensitive phenotype conferred by N-terminal truncated protein (1-47), as discussed in Chapter 4. However, to conclude the complementation experiment, it is necessary to test the stability of SKIP deletion derivatives in yeast cells by Western analysis of the GBD-tagged SKIP peptides.

On the other hand, in the Prp46p two-hybrid screens all the preys are fused at their N-terminal end with the Gal4p-transactivation domain, which provided the N-terminal region (Albers *et al.*, 2003). Similarly, co-immunoprecipitation experiments with $^{35}$S-labelled Prp46 truncated forms and His$_6$-tagged Prp45p mapped the region of interaction in Prp46p within the portion identified in two-hybrid screens (amino acids 127-432) plus the N-terminal end (Albers *et al.*, 2003). It would be interesting to determine if the Prp45p-Prp46p direct interaction is maintained for the human homologues of these proteins, SKIP and PRLG1, and also whether the N-terminal domain of SKIP contributes to these interactions.

Additional two-hybrid screens were performed recently at Hybrigenics using Prp46p as a bait (A. Diment, unpublished data). The results not only confirmed that the main interactor of Prp46p is Prp45p, but also trimmed down the previously found Prp45p portion necessary for this interaction (amino acids 54-204) by around 110 amino acids (residues 55-98) (see Figure 3.1.A). The interaction with Prp46p in a yeast extract proved to be stable and strong, as shown by increasing the stringency of
co-immunoprecipitation conditions, i.e. the salt concentration (NaCl) from 150 mM, as above, to 500 mM. These results are presented in Chapter 5 (section 5.4).

The requirement for Prp45p in the formation of the active spliceosome is in agreement with data obtained for its human homologue, SKIP, for which it was determined that it enters precisely at the time of spliceosome activation after formation of complex B (inactive spliceosome stage) but before complex C (active spliceosome). This requirement was also determined for other members of the yeast Cwc-protein complex like Prp19p (Tarn et al., 1993), Snt309p (Chen et al., 1998), Cef1p (Tsai et al., 1999), Ntc20p and Ntc30p (Chen et al., 2001), i.e. they associate with the spliceosome simultaneously or just after the dissociation of U4 snRNA, step in the assembly process that marks the transition from complex I/B to functional spliceosome (complex II/C).

Prp45p synthesized either in *E.coli* or in mammalian reticulocyte lysates partly reconstitutes the splicing activity in the Prp45p-depleted extract. Complementation with different range of protein concentrations did not lead to a better complementation, which demonstrated that Prp45p is not the only limiting factor for the reaction. Initial experiment with recombinant protein gave the same phenotype, i.e. inefficient reconstitution of the Prp45p-depleted splicing activity (M. Albers, PhD Thesis) possibly explained by the improper folding or post-translational modification of the protein produced in *E.coli*. This situation is similar with the ones observed for other members of Prp19/Ntc-protein complex: Prp19p (Tarn et al., 1994) or Snt309p (Chen et al., 1999) that failed to complement the Prp19p /Snt309p-immunodepleted extracts. However the splicing activity was restored in these cases by the addition of the entire Prp19p-associated complex. Therefore it is possible that this protein complex functions as a whole and the depletion of one of its components triggers the inactivation of other(s). Indeed, *in vitro* depletion of Cef1p from cell extracts by the addition of anti-Cef1p antibodies specifically inhibited the association to the spliceosome of the other components of Ntc/Cwc-protein complex (Tsai et al., 1999).
Chapter 4  Construction of prp45 Temperature-sensitive Mutants
Chapter 4: Construction of prp45 Temperature-sensitive Mutants

4.1 Introduction

In order to understand the function of PRP45 in S. cerevisiae, temperature-sensitive (Ts') lethal mutants of this gene were created and analysed.

Chromosome I of S. cerevisiae where PRP45 is located has been extensively studied in order to identify essential genes and to characterize their physical and genetic organisation (Coleman et al., 1986; Steensma et al., 1987; Diehl and Pringle, 1991). For these purposes two approaches were used. Firstly, molecular analyses by northern blotting of transcripts encoded by different segments of chromosome I, and gene-replacement/complementation experiments led to the identification of the essential gene PRP45 (Diehl and Pringle, 1991).

Secondly, various attempts to obtain temperature-sensitive mutations of the genes on this chromosome failed to identify a Ts' allele of PRP45. Three different approaches were used to try to get Ts' mutations on this chromosome. In 1984, Kaback et al. mutagenized a strain monosomic for chromosome I, using either ethyl methanesulphonate (EMS) or N-methyl-N'-nitro-N-nitrosoguanidine (NG) as mutagen. In this way 32 independent mutations that mapped to chromosome I were isolated, and localised to three genes. When the study was repeated using different mutagens like ultraviolet light and nitrogen mustard (Harris and Pringle, 1991), nearly all of the mutations obtained fell into the same few genes. A more specific, third attempt was made by Harris et al., 1992. This study tried to generate Ts' lethal mutations in three genes that had not been affected during the previous Ts' mutant hunts, but had been identified and shown to be essential during molecular analyses of chromosome I. One of these three genes was FUN20 or PRP45. Briefly, this approach involved passing a plasmid containing PRP45 through a mutD mutator strain of E. coli, followed by screening for Ts' lethal mutations using the plasmid shuffle technique. As all types of base substitutions are induced at high frequencies in the mutD strain (different from the first two methods which yield only a limited number of amino acids substitutions) this approach should generate more mutants. Unfortunately, the results suggested PRP45 could not yield Ts' lethal mutations. The
situation was the same for one of the other genes analysed in the same study (FUN12) while in the case of the third gene, FUN53, they succeeded in recovering Ts\(^{-}\) lethal alleles but only at a very low frequency. As the sequencing analysis done during this study revealed one single base-pair substitution within the Ts\(^{-}\) alleles obtained, the authors conclude that “many of the single-copy essential genes on chromosome I cannot be rendered temperature-sensitive by single or simple combinations of substitutions” and “the genes that can yield Ts\(^{-}\) lethal alleles by single substitutions do so at extremely low frequency”.

In the present work a more specific mutagenesis of PRP45 was carried out, with a rate of mutagenesis higher than in the other studies, an approach that yielded temperature-sensitive alleles of this gene. As concluded above, the mutations found were located in two regions of PRP45 within its SNW domain. These mutations were re-created within the wild-type gene by site-directed mutagenesis in order to identify which mutation is responsible for the temperature-sensitive phenotype. In this chapter, analysis took the form of examination of growth rates at permissive and non-permissive temperatures.

4.2 Random mutagenesis of PRP45

4.2.1 Random PCR mutagenesis

The strategy for mutagenesis used in this work is based on the skewed nucleotide PCR method described in the report of Leung \textit{et al.}, 1989. In this protocol (section 2.3.2.12), random mutations were generated within PRP45 using a modified PCR procedure. In order to get a high rate of mutagenesis consisting of both transitions and transversions, the dATP concentration added to the reaction mixture was five times lower than the concentration of the other three deoxynucleotides (dCTP, dGTP and dTTP). In this way one of the over-represented deoxynucleotides was occasionally misincorporated instead of dATP into the extended DNA strand. During the amplification steps these misincorporations were propagated as well as new
errors being created.

Initially, a plasmid that provided the template for mutagenic PCR was created. This plasmid, named pRS-45 was engineered by cloning the entire chromosomal \textit{PRP45} gene flanked by 500 nucleotides from its promoter region (P) and by 500 nucleotides from its terminator region (T), into the centromeric pRS314 vector. Oligos PS&5-45M and TXHO-45M (Table 2.7) that annealed in P region and T region, respectively, were used as primers for skewed nucleotide PCR (Figure 4.1). Then, the mutagenized PCR product was co-transformed with a linearised plasmid, pRS-PT, that contains P and T regions in a \textit{PRP45} conditionally regulated strain, YMA45/2.

4.2.2 Construction of pRS-PT vector

Plasmid pRS-PT was created in a 3-way ligation. Firstly 0.5 kb containing the promoter region of \textit{PRP45} (P region) and 0.5 kb with the terminator region of the same gene (T region) were amplified from chromosome I by PCR. The primers used contain restriction sites at the 5’ ends so that, when joined, the P region and T region are separated by an NcoI restriction site and can be ligated in the centromeric plasmid pRS314 (\textit{TRPI} is its selectable marker). Plasmid pRS314 was cut with \textit{SpeI} and \textit{PstI}, the P region was digested with \textit{SpeI} and \textit{NcoI} and the T region digested with \textit{NcoI} and \textit{PstI}. Then the three fragments were ligated and the resulting plasmid was designated pRS-PT. Prior to transformation pRS-PT was linearised by digestion with \textit{NcoI}.  

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Figure 4.1 Schematic representation of the four screens performed to identify Ts' mutants of PRP45.
Oligonucleotide primers PS&5-45M (S) and TXHO-45M (X) were used in a skewed nucleotide PCR to amplify PRP45 ORF (together with its Promoter (P) and Terminator (T) regions) from plasmid pRS-45. Five hundred nanograms of mutagenised PCR product and 250 nanograms of NcoI/BsmI digested pRS-PT plasmid were co-transformed in PRP45 conditionally regulated strain, YMA45/2, and plated on either YMGRS -His, -Trp or YMM -His,-Trp media. Four days after transformation the cells were replica plated onto YMM-Trp (Glc-Trp) at different temperatures to select for temperature-sensitive or cold-sensitive mutants and then verified by re-streaking the Ts' candidates on YPDA. From the cells grown at 23°C that were defective at 37°C, the pRS-45-derived plasmids were isolated (plasmid rescue procedure) and their identities checked by digestion with PvuII. Then they were re-transformed in YMA45/2, the transformants selected directly on YMM-Trp plates and the Trp' colonies re-streaked in duplicates onto YPDA plates and grown at 37°C and 23°C for 3 days.
**Mutagenised PCR product**

**Linearised pRS-PT**

**Skewed nucleotide PCR**

39,705 transformants replica plated on Glc-Trp

- **37°C**
- **23°C**
- **16°C**

93 Ts candidates

<table>
<thead>
<tr>
<th>1) 62 double-checked Ts candidates.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2) rescue the plasmids and check them through restriction-digestion.</td>
</tr>
<tr>
<td>3) transform the rescued plasmids in YMA45/2 to re-examine the growth defect at restrictive temperatures</td>
</tr>
</tbody>
</table>

5 Ts alleles of **PRP45**
4.2.3 Co-transformation of \( \text{PGAL: PRP45} \) conditionally regulated strain

The transformed YMA45/2 (Albers et al., 2003) cells with randomly mutagenized \( \text{PRP45} \) gene and the linearised pRS-PT were plated or replica plated (in case that the initial plating was done on galactose-based media) on glucose – Trp (YMM – Trp). In this way the chromosomal \( \text{PRP45} \) is turned off and the cells rely on the episomal copy which is “provided” if the homologous recombination occurred between the mutagenized \( \text{PRP45} \) and \( \text{NcoI} \) digested pRS-PT (Figure 4.1).

In order to optimise the transformation efficiency and the selection of transformants, small-scale transformation experiments were done to figure out the optimum ratio between the PCR product and linearised vector. The number of transformants was greatest when YMA45/2 strain was transformed at an \( \text{OD}_{600} \) of 0.6 with a ratio of 500 ng PCR product: 250 ng linearised vector. Furthermore, when a negative control of \( \text{NcoI-digested pRS-PT} \) was included in transformation, \( \text{Trp}^+ \) transformants were recovered on selective plates that indicated that the digestion was not complete. This situation was overcome by double digestion of pRS-PT with \( \text{NcoI} \) and \( \text{BsmI} \), the latter having a unique restriction site situated in the T region 28 bases down-stream of the \( \text{NcoI} \) site.

Five randomly picked Trp\(^+\) colonies were had their plasmids rescued (section 2.3.2.2) and sequenced to determine the frequency of mutations. The mutagenesis rate was 0.5\% (28 mutations in 5700 bp). This figure was lower than the value of 1.0\% reported by Leung et al., 1989, but presented an advantage when identifying the mutations responsible for the Ts phenotype. Most point mutations examined represented transitions A:T to G:C and T:A to C:G, figures that are in concordance with the fact that dATPs were significantly less incorporated during mutagenic PCR. The figure of 0.5\% that represents 6 changes per 1.1kb (the size of \( \text{PRP45} \)) is higher than 0.16\% obtained by Harris et al., 1992, when mutagenizing \( \text{FUN53} \) (see section 4.1). The rate of mutagenesis of \( \text{PRP45} \) in the same study was not reported.
4.2.4 Screening for Ts' lethal mutations

After transformation, the cells were grown on -Trp plates (YMGRS-Trp or YMM-Trp) at 23°C for 3 days, at which point the colonies were counted and replica plated a few times onto glucose (YMM-Trp) to ensure that the chromosomal copy of \textit{PRP45} was completely switched off. This was indicated by the negative controls: YMA45/2 cells transformed with plasmids pRS314 and pRS-PT that do not contain the \textit{PRP45} gene (see Figure 4.1).

Four Ts' screens designated Primo, Secundo, Terto and Quatro were performed that differ from each other in terms of cell density plated onto -Trp plates, selection and number of replica platings done. The characteristics and the results of these screens are shown in Table 4.1.

After the selection, the transformants were replica plated onto YMM-Trp plates and incubated at 14°C (for 6 days), 37°C (for three days) and 23°C (for 4 days). The latter replica plating was done last to ensure that the putative temperature-sensitive mutants did not result from poor transfer of cells during this procedure. Transformants able to grow on YMM-Trp at 23°C but not at 37°C were scored as presumptive Ts' mutants. In these screenings 93 temperature-sensitive candidates and no cold-sensitive mutants there were identified.

All the putative Ts' mutants were re-checked by streaking 2 colonies from each candidate from the YMM-Trp (23°C) plate onto YPDA plates at 37°C and 23°C alongside positive control strain YMA45/2 containing wild-type \textit{PRP45} in the same vector (pRS-45). This analysis at different temperatures was performed on YPDA in order to avoid the selective pressure of -Trp media on the mutants and to optimise the growth of the cells. After 3 days on YPDA, 31 clones displayed a similar growth to the wild-type at non-permissive temperature and therefore they were discarded. The remaining 62 candidates with the growth completely abolished at 37°C had the plasmids rescued and verified by restriction digestion (data not shown).

In order to confirm that the Ts' lethal phenotype was plasmid dependent, these vectors were re-transformed into strain YMA45/2, transformants plated directly onto YMM-Trp plates and, after 4 days of growing at permissive temperature (23°C), the positive Trp' clones had the Ts' analyses re-done as before on YPDA (Figure 4.2.A).
Table 4.1 Summary of the *PRP45* Ts' screens

<table>
<thead>
<tr>
<th>Screen</th>
<th>Selectiona</th>
<th>No. of transformants screened</th>
<th>1st replica platingb</th>
<th>2nd replica platingb</th>
<th>replica plating @ different °Cc</th>
<th>Putative Ts' mutants</th>
<th>Ts' after checking in duplicate on YPDAd</th>
<th>Re-analysede</th>
<th>Ts' mutants</th>
<th>Ts' mutants' nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primo</td>
<td>YMGRS-Trp, His</td>
<td>13,140</td>
<td>YMM-Trp, His</td>
<td>YMM-Trp, His</td>
<td>YMM-Trp</td>
<td>11</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>P-1</td>
</tr>
<tr>
<td>Secundo</td>
<td>YMGRS-Trp, His</td>
<td>11,592</td>
<td>YMM-Trp</td>
<td>YMM-Trp</td>
<td>YMM-Trp</td>
<td>15</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>S-57 (prp45-57)</td>
</tr>
<tr>
<td>Terto</td>
<td>YMM-Trp</td>
<td>7,453</td>
<td>-</td>
<td>-</td>
<td>YMM-Trp</td>
<td>34</td>
<td>16</td>
<td>16</td>
<td>1</td>
<td>T-113 (prp45-113)</td>
</tr>
<tr>
<td>Quatro</td>
<td>YMM-Trp</td>
<td>7,520</td>
<td>-</td>
<td>-</td>
<td>YMM-Trp</td>
<td>33</td>
<td>33</td>
<td>16</td>
<td>1</td>
<td>Q-9</td>
</tr>
</tbody>
</table>

a The medium the cells were plated on after transformation.
b The replica platings necessary to completely switch off the chromosomal *PRP45* gene controlled by *P*$_{GAL}$ promoter.
c Replica plated on YMM-Trp and incubation at 14°C, 37°C and 23°C.
d The putative Ts' mutants were checked by re-streaking each in duplicate on YPDA and incubating at 14°C, 37°C and 23°C.
e Strain YMA45/2 was transformed with the plasmids containing *PRP45* mutants to check the maintenance of the phenotype.
4.2.5 Sequencing the Mutants

The confirmed *prp45* Ts' mutants were sequenced (section 2.3.2.15). In comparison with the wild-type *PRP45* sequence, the nucleotide exchanges and the corresponding amino-acid alterations within the Prp45 protein were mapped and the results are presented in Figure 4.2.13.

As can be seen, the Primo and Quatro Ts' mutants are truncated forms of Prp45p that retained the NH$_2$-terminal end of the protein. The shortest one, P-3, which consists only of the first 47 amino-acid residues of Prp45p, supported growth at 23°C but not at 37°C. This result is contradictory with the report of Martinkova *et al.*, 2002, who found that truncated Prp45p (1-119) is incompatible with the survival of cells, while the first 190 amino-acids are sufficient for growth even at 37°C. Similarly, mutant P-1, spanning the first 142 amino acids is viable and does not contain the SNWKN motif. An interesting situation concerns the mutant Q-9, that is also a truncated form of Prp45p, with 186 amino acids, and is similar in length to P-5, a Primo candidate found initially to be Ts' mutant but, as Figure 4.2.A. illustrates, did not maintain the phenotype after re-transformation. Both P-5 and Q-9 contain frame-shift mutations downstream of the SNWKN motif at positions 179 (for P-5) and 176 (for Q-9), which led to the premature termination of the coding region (Figure 4.2.B). However, Q-9 contains additional substitutions (especially a mutation of the conserved position 164 in front of SNWKN motif (lysine to glutamate)) that perhaps enhance the Ts' phenotype. The P-8 mutant that lacks the C-terminal 38 amino acids was previously found as a putative Ts' candidate in the Primo screen and subsequently showed a growth rate at 37°C similar to the wild-type. This is compatible with the results of Martinkova *et al.*, 2002, who showed that the last 49 amino-acid residues of Prp45p were dispensable for growth at 37°C.

The remaining temperature-sensitive mutants, S-57 (or *prp45*-57) and T-113 (or *prp45*-113) were chosen as the main objects of further study. There are many reasons behind this decision: first of all, given the number that have been checked on plates, only these were very stable regarding the Ts' phenotype.
Figure 4.2. Schematic diagram summarizing the phenotype and mutations generated by random PCR mutagenesis in PRP45.

A. pRS-45-derived plasmids carrying prp45 Ts' alleles (P-1, P-3, Q-9, S-57 and T-113) and wild type (WT) were re-transformed in GAL-regulated PRP45 strain, YMA45/2, to check for the reproducibility of the Ts' phenotype. Mutant P-5 (similar in length to Q-9) and mutant P-8 (similar to a previous study) were analysed alongside. The transformed cells were plated onto glucose-based media lacking tryptophan. The Trp+ cells were re-streaked onto YPDA plates and incubated at 23°C and 37°C for 3 days before being photographed.

B. Wild-type Prp45p is illustrated as a bar with N-terminal amino acid at position 1 and C-terminal amino acid at position 379 and below are shown the mutant protein products. The regions A and B are shown in green as well as the amino acid position they range within. The highly conserved SNWKN motif is depicted as a blue vertical bar. The substituted amino acids and their positions are shown; conservative changes shown in red and semi-conserved are orange. (*) represents a frame-shift mutation and STOP indicates nonsense mutations that give rise to the truncated proteins (P-1 and P-3). The names of these protein products are shown on the right.
Secondly, the mutations within these alleles are in two conserved regions of Prp45p, located in the SNW domain, flanking the SNWKN motif. For ease of characterisation, these regions were denominated \( A \), the one situated upstream of SNWKN motif, spanning amino acids 120 – 136 and, \( B \), situated downstream of SNWKN motif between amino acids 190 and 211.

Within region \( A \), both mutants share the same change in residue 130 (lysine to glutamate), which was found in other sequenced Ts\(^-\) mutants including T-104 where this was the only mutation but did not determine the Ts\(^-\) phenotype. Lysine at position 130 is moderately conserved among the homologues of Prp45p, being substituted with another positively charged amino-acid, arginine, in \( D. \ melanogaster \) and \( S. \ pombe \). Apart from this, the \( prp45-57 \) allele contains a change in position 135: the semi-conserved amino-acid valine to glutamate while the \( prp45-113 \) mutant has a mutation affecting the highly conserved lysine (position 133) substituted by a glutamate. Thus the positively charged consensus sequence 103KKARKVV109 of region \( A \) becomes less charged due to the presence of two negatively charged amino acids.

Regarding region \( B \), the changes are not so dramatic: \( prp45-57 \) has an asparagine to aspartic acid substitution in position 192, a highly conserved leucine (position 208) changed into serine, and asparagine (position 210) into aspartic acid. As for \( prp45-113 \), there is only one mutation affecting the semi-conserved leucine in position 204 that changes to proline.

These conditional mutants along with the wild-type strain were grown in liquid culture (YPDA) at 23\( ^\circ \)C and 37\( ^\circ \)C and the growth rates were monitored by measuring the optical density (OD\(_{600}\); section 2.2.8). As depicted in Figure 4.3, at 23\( ^\circ \)C there is no difference between the cell growth of mutant strains \( prp45-57 \) and \( prp45-113 \) and of wild-type strain (\( PRP45 \)).
Chapter 4: Construction of prp45 Temperature-sensitive Mutants

Figure 4.3. Growth curves of the Ts° mutants prp45-57 and prp45-113 alongside wild-type (PRP45).
Overnight cultures of these strains were grown at 23°C in YPDA to mid-logarithmic phase and aliquots were used to inoculate 50 ml of YPDA at 23°C (upper panel) or 37°C (bottom panel) to OD_{600} of 0.1. Aliquots were removed every hour and the OD_{600} was read. Upon reaching OD_{600} of 0.7, the cultures were diluted (with media pre-warmed at the appropriate temperature) to maintain log growth.
When the temperature was shifted to 37°C, growth of the mutant strains slowed after 2 hours and was arrested after 8 – 9 hours at the restrictive temperature. Overall, this experiment validates the results obtained on solid media that show that the prp45 mutants have growth defects at elevated temperature.

4.3 Site-directed Mutagenesis of PRP45

In order to understand which of the mutations obtained by random mutagenesis was responsible for the Ts' phenotype, i.e. to identify residues essential for Prp45p function, site-directed mutagenesis was performed.

All the individual mutations obtained in both Ts' mutants prp45-57 and prp45-113 were re-created separately in the wild-type gene using the two-stage PCR overlap extension method. Plasmid pRS-45 (CEN TRP1 PRP45) was used as the template for the first amplification step as described in section 2.3.2.13.1. Residues targeted for mutagenesis were situated in region A, B or in both of these. The mutated DNA products of the second-step amplification were digested with restriction endonucleases and inserted into pRS-45 in lieu of the corresponding wild-type restriction fragments. These constructs were sequenced before being transformed into the PRP45 conditionally regulated strain, YMA45/2 to check for the Ts' phenotype.

Figure 4.4 shows all the changes inserted to re-build the mutations found in prp45-57, in so-called region A - 57A (K130E, V135E), region B - 57B (L208S, N210D), 57B1 (L208S), 57-201 (N192D) and in A and B - 57AB2 (K130E, V135E and N210D) and 57AB (K130E, V135E and L208S). Similarly, for mutations found originally in prp45-113: in region A – 113A (K130E and K133E), in B - 113B (L204P), at position 273 - 113C (L273P) and AB - 113AB (K130E, K133E and L204P).
Figure 4.4. Amino acid substitutions within Prp45p created by site-directed mutagenesis. The amino-acid sequence of Prp45p is schematically shown as a horizontal bar ranging from residue 1 (N-terminus) to residue 379 (C-terminus) and so-called conserved regions A and B are depicted in green and the highly conserved SNWKN motif is illustrated as a blue vertical bar. prp45-57 and prp45-113 are the temperature-sensitive (ts) mutants found in random mutagenesis screens, while the others, 57A to 57AB and 113A to 113AB are mutant alleles of PRP45 generated by site-directed mutagenesis (SDM). The conserved amino acids are depicted in red and the semi-conserved ones in orange, and their corresponding position in the wild-type Prp45p is shown. After transformation in YMA45/2, only 57AB and 113AB mutants kept the Ts' phenotype. The extra mutations in constructs 57-192-9 and 113-C are errors generated during amplification and cloning.
Incubating the transformants at 37°C on glucose-based media (i.e. when the chromosomal PRP45 allele is shut off and the viability of the cells depends on the presence of the plasmid) showed that only mutants 57AB and 113AB which have the conserved/semi-conserved amino acids substituted in both regions, conferred the temperature-sensitive phenotype to the host cells (Figure 4.5).

\[ \text{\textbf{Figure 4.5 Growth of Prp45p mutants generated by site-directed mutagenesis at permissive and non-permissive temperatures.}} \]

The plasmids containing wild-type PRP45 (pRS-45) and the mutant prp45 constructs p57A, p57AB, p113AB depicted in Figure 4.4 were transformed into YMA45/2 and plated on Glc-Trp. After four days at 23°C one colony from each transformant was re-streaked on YPDA and incubated for 3 days at 23°C (permissive temperature) and at 37°C (restrictive temperature) before being photographed.
All the constructs generated by site-directed mutagenesis were thoroughly sequenced. During the SDM some un-wanted mutations were found in constructs 57-201 and 113C, probably generated during the PCR by Taq polymerase. The use of this enzyme was discontinued and the proofreading Pfu polymerase was utilized in subsequent work.

This data not only confirm the theory of Harris and Pringle, 1992, which stipulated that more than one mutation is necessary to get temperature-sensitive alleles of PRP45 but also showed that the two conserved domains A and B are essential for the Prp45p function.

4.4 Construction of c-Myc tagged PRP45 / prp45 strains

In order to rigorously show that the mutations within the prp45 gene caused the temperature-sensitive phenotype, chromosomal tagged prp45 Ts' alleles were generated. This would allow further investigation of the consequences of these prp45 mutant alleles on the splicing and / or transcription processes. At the same time the tagged alleles (wild-type and mutant) would represent a tool for the examination whether the Ts' phenotype is due to the loss of function of Prp45 mutant proteins or is a result of the instability of these proteins.

First of all, a tagged version of the wild-type PRP45 was constructed in the pRS-45 plasmid. Using site-directed mutagenesis by whole plasmid amplification (section 2.3.2.13.2), a unique NcoI restriction site was generated exactly at the last codon of the PRP45 open reading frame. Thus, the TAG stop codon of PRP45 was changed into ATG and the sequence become CCATGG, which represents the NcoI restriction site (Figure 4.6). The plasmid created was referred as to pRS-45N.
1) Site-directed mutagenesis by whole plasmid amplification was used to introduce an \textit{NcoI} restriction site in lieu of the STOP codon, \textit{TAG}, of \textit{PRP45} ORF and thus a new plasmid, pRS-45N was created. 2) Oligonucleotide primers c-Myc-TRP-Nco-F and c-Myc-TRP-Nco-R were used to amplify a cassette from plasmid pFA6a-13Myc-TRP1 of approximately 1.8 kb containing 13 c-Myc epitopes (\textit{13 Myc}) fused with \textit{ADH1} terminator (the black box) and \textit{TRP1} marker gene (\textit{TRP1}). 3) The amplified cassette was then cut with \textit{NcoI} (N) and ligated into plasmid pRS-45N, previously linearised by \textit{NcoI} (N) digestion, to produce plasmid pMM45. 4) A DNA fragment of 2.3 kb that contains this cassette and the 500 bp of the \textit{PRP45} terminal region (T) was isolated from pRS-45N by digestion with \textit{SfoI} (S), located upstream of \textit{NcoI} site, and \textit{XhoI} (X). 5) This fragment was then ligated into p57AB and p113AB, previously cut with \textit{SfoI} and \textit{XhoI}, producing plasmids pMM57 and pMM113. Restriction digestion and DNA sequencing were used to check the identity of the newly created plasmids.
The manufactured restriction site allowed the integration downstream of PRP45 ORF of a cassette of 1.8 kb, containing a sequence that encodes thirteen c-Myc epitopes followed by the terminator region of ADH1 gene and TRP1 marker gene. The cassette was amplified from vector pFA6a-13Myc-TRP using oligonucleotide primers c-Myc-TRP-Nco-F and c-Myc-TRP-Nco-R that contain NcoI restriction sites in the 5’end. The PCR product was gel purified, digested with NcoI and ligated into NcoI digested pRS-45N. In this way the PRP45 open reading frame lacking the STOP codon was fused in-frame to the DNA sequence of the c-Myc tag. This new plasmid was called pMM45.

To tag the prp45 Ts’ mutants, 57AB and 113AB, the cassette was excised from pMM45 by restriction digestion with SfoI and XhoI. SfoI has a unique restriction site 4 nucleotides upstream of the 3’end of PRP45 ORF, while XhoI was used to construct pRS-45 at the 3’end of the PRP45 terminator region. The SfoI / XhoI restriction fragment was ligated into SfoI / XhoI digested plasmids p57AB and p113AB. Thus the two prp45 mutant alleles, 57AB and 113AB become tagged with c-Myc. The newly created plasmids were called accordingly: pMM57 and pMM113. Subsequently, the complete PRP45–c-Myc coding sequence with 0.5kb of upstream and downstream DNA were amplified from plasmid pMM45, gel purified and transformed into the haploid yeast strain BMA38a (Figure 4.7). The 0.5 kb flanking regions were included in order to allow homologous recombination of the PCR product with the chromosomal PRP45 gene and thus, its replacement with the tagged gene (see section 2.2.7). As the c-Myc cassette contains the TRP1 marker, the selection of transformants was done onto medium lacking tryptophan (YMM-Trp). After 4 days at 23°C, tryptophan prototrophic transformants were obtained. This strain was referred to as YMM45.

The same protocol was applied for pMM57 and pMM113 to create chromosomal c-Myc tagged versions of prp45 mutants 57AB and 113AB. The mutants were amplified and transformed into strain BMA38a with selection for Trp+ transformants to produce strains YMM45-57 and YMM45-113.
Figure 4.7 Construction of c-Myc-tagged Prp45p strains, YMM45, YMM45-57 and YMM45-113.

A. PCR products containing PRP45-c-Myc-TRP1 or 57AB-c-Myc-TRP1 or 113AB-c-Myc-TRP1 flanked by 0.5kb sequences homologous to the sequences upstream and downstream of the PRP45 ORF, were used to replace the chromosomal copy of PRP45 gene in BMA38a haploid yeast strain. The amplifications were done on pMM45, pMM57 and pMM113 using the primers PS&5-45M (1) and TXHO-45M (2). B. PCR on cells from strains YMM45, YMM45-57 and YMM45-113 as well as wild-type BMA38a as control. The annealing positions of the primers, 3 and 4 are shown (in panel A.) as well as the length of the amplified DNA.
A.

1. PRP45 13My T

2. pMM45/ pMM57/ pMM113

PCR and Transformation

3. PRP45 13My T

Homologous recombination on Chromosome 1

4. PRP45 13My T

Chromosome 1

B.

BMA38a

YMM45/ YMM45-57/ YMM45-113

M BMA38a YMM45 YMM45-57 YMM45-113 -DNA

2.4 kb
The integration of the \textit{prp45} alleles into the chromosomal locus was verified by PCR on one of the yeast colonies from each strain constructed (Figure 4.7.B). The oligonucleotide primers used were PRP45-522-F and 13-Myc-R. A single product of the expected size of 2.4 kb was amplified from the transformed cells but not from the wild-type strain, suggesting that the integration occurred at the desired locus.

The cloned alleles were thoroughly sequenced to confirm not only their correct integration but also to exclude the acquisition of unwanted mutations during amplification or cloning (Figure 4.8). At the same time this step confirmed the presence of the desired mutations in YMM45-57 and YMM45-113 as they were generated for 57AB and 113AB.
Figure 4.8 Schematic representation of DNA sequences of YMM45, YMM45-57 and YMM45-113. A. The *PRP45* ORF in yeast haploid strains YMM45, YMM45-57 and YMM45-113 was sequenced using the oligonucleotide primers: S (PS&5-45M), P (PRP45-F-PROMOTER), A (PRP45-F1), B (PRP45-F2), M (13-Myc-R), N (c-Myc-TRP-Nco-R) and X (TXHO-45M). The annealing position of these primers is also shown. B. and C. Sequencer TM4 graphs showing the results of the sequencing analysis within the regions A and B of YMM45-57 and YMM45-113. YMM45 alignment in the same regions represents the control. The position number of the modified nucleotides is shown at the top and the mutations are enclosed in red squares.
4.5 YMM45-57 and YMM45-113 present a growth defect at non-permissive temperature

Having confirmed the presence of the desired mutations in these strains, it was of interest to test their ability to grow at the restrictive temperature. The Trp\(^+\) transformants from which the DNA sequences were analysed were streaked on YPDA medium and growth of the mutant strains YMM45-57 and YMM45-113 at 23°C and 37°C was compared with wild-type strain (YMM45) grown under the same conditions (Figure 4.9.A). As with the results previously obtained when analysing the same mutants on plasmids (section 4.3), both YMM45-57 and YMM45-113 present temperature-sensitivity at 37°C, with the latter having a stronger Ts\(^+\) phenotype. This demonstrates that the mutations created in \(PRP45\) are indeed responsible for the growth defect at the non-permissive temperature.

This was further confirmed by growth of these three strains in YPDA liquid medium at 23°C and 37°C. By comparing these growth rates at 23°C to the ones obtained for untagged \(PRP45\) alleles (wild-type and mutants) presented in Figure 4.3, one can conclude that there is no difference between them, the doubling time being 2 hours in both situations. Thus the addition of the c-Myc tag at the C-terminal of Prp45p alleles does not affect the function of these proteins under these conditions. However, at 37°C, growth starts to slow down after 5 hours for YMM45-57 and after 1 hour for YMM45-113, and then is arrested after about 10 and 6 hours, respectively.
Figure 4.9 Growth of mutant strains YMM45-57 and YMM-113 at permissive and restrictive temperature. A. *PRP45* yeast strain, YMM45 and the derivate mutants with temperature-sensitive *prp45* alleles, YMM45-57 and YMM45-113 were streaked on YPDA plates and incubated for 3 days at 37°C and 4 days at 23°C. B. The same strains were grown overnight in YPDA liquid media at 23°C to mid-logarithmic phase before their growth was monitored at 23°C and 37°C. Aliquots were removed every hour and the optical density of the cells (OD<sub>600</sub>) was measured.
A.

23°C

YMM45-57

YMM45-113

37°C

YMM45

YMM45-113

B.

23°C

37°C

100

10

1

0

1

2

3

4

5

6

7

8

9

10

Time (hours)

OD₆₀₀

YMM45-57

YMM45-113

Time (hours)

OD₆₀₀

YMM45

YMM45-113

Time (hours)
The reason why these two mutants are behaving slightly differently at 37°C, perhaps relies on the differences between the amino acid residues mutated in region A: two conserved lysines changed in YMM45-113 versus one conserved lysine and one semi-conserved valine in YMM45-57. It is possible that the positively charged region A (with the consensus sequence RFVGKKARKVVA) is involved in forming salt bridges with a negatively charged protein sequence, or in nucleic acids binding. The presence of two extra negatively charged amino-acid residues (glutamate) may disturb these interactions, more in YMM45-113 where two lysines are affected and the sequence becomes less positively charged. As for region B, two leucine residues are affected in both mutants, in YMM45-113 one being changed with a proline residue. Proline is a cyclic amino acid that usually creates a turn in the amino-acid chain with consequences for protein folding.

4.6 Discussion

Previous studies of chromosome I of S. cerevisiae and especially of PRP45 suggested that this gene is difficult to get Ts' mutants regardless of the mutagen used. Indeed, the use of mutD E.coli strain, defective in the proof-reading activity of DNA polymerase proved to produce a high rate of spontaneous mutations, around $10^4$ more than the wild-type but not for PRP45 or its neighbours: FUN12 and FUN53.

Here, using a PCR-based random mutagenesis protocol, 63 Ts' mutants were obtained. When 45 of these mutants were re-analysed, only 5 seemed to preserve the Ts' phenotype. This discrepancy could be due to some un-wanted recombinational events that took place initially not only between the mutagenized PCR product and the lineariised plasmid, but also with the chromosomal copy of PRP45. So the initial defect observed was in fact due to cooperation between the mutations located on the plasmid and on the chromosome. A better strategy would have used a PRP45 knockout strain (to avoid any subsequent recombination) that hosts a URA3–based plasmid carrying the wild-type PRP45. The mutagenized PCR product and the
linearised plasmid should have been transformed as before and the screen for Ts' mutants performed using plasmid shuffle technique on 5-FOA plates.

Three of the five Ts' mutants are truncated forms of Prp45p that contain the N-terminal region of the protein up to at least 47 amino acids. The N-terminal region of Prp45p (1-47) does not contain significant stretches of amino acids nor conserved residues among the other Prp45p homologues but proved to have an important role for the protein folding and/or function. First, it seemed to be important for cell viability: a truncated form of Prp45p containing the N-terminal 190 amino acids supports cell growth in a haploid strain while the same fragment without the first 53 amino acids is incompatible with cell survival (Martinkova et al., 2002). A second line of evidence is given by the results presented in Chapter 3 and Annex I regarding the interaction of Prp45p with Prp46p. Co-immunoprecipitation experiments showed that the N-terminal region of Prp45p is essential for the in vitro interaction with Prp46p while the minimum region of interaction found in two-hybrid screens (54-208) is not sufficient in vitro. However, before drawing any conclusion it is necessary to fully analyse this truncated protein in another background, as YMA45/2 host strain seemed to pose problems and to affect the phenotypes determined by the prp45 mutants located on plasmids.

Another interesting trait of the mutants is that certain sites of the PRP45 gene are susceptible to mutagenesis. Regions A and B seemed to have more than one mutation; an identical A:T to G:C transition in position 87 (resulting in a silent mutation) as well as the A:T to G:C in position 388 (K104E) were found in many independent mutants. This clustering was also observed in the early study (Harris et al., 1992) for FUN53 where mutations that determined the Ts' phenotype are preferentially located in a certain region. These regions do not contain stretches of adenine that could have determined the substitutions and perhaps there are more relaxed and prone to mutagenesis due to the nucleotide context or constraints in DNA repair.
Chapter 5  
Analysis of prp45 Ts− Mutant Proteins
5.1 Introduction

The availability of the tagged versions of temperature-sensitive alleles of PRP45 allowed the study of the functions and interactions of the encoded proteins. To date, two different roles for Prp45p have been proposed and demonstrated: as a splicing factor in yeast (Albers et al., 2003) and as a co-regulator in many transcription pathways in mammalian cells (Zhou et al., 2000, Leong et al., 2001, Zhang et al., 2001).

By making use of the two Ts- mutants described previously, the work presented in this chapter attempts to shed more light on the relationship between the structure and the function of Prp45 protein.

5.2 prp45 Ts- mutant proteins are stable at non-permissive temperatures

As the wild-type and the mutant alleles of the PRP45 gene were tagged, the next step was to examine the proteins encoded by them. Protein samples were taken at different time points after the shift to 37°C when, according to the growth curve experiment (Figure 4.9) the growth started to slow, and when it levelled off. Total protein extracts were prepared from similar numbers of cells (3 OD₆₀₀ units; as described in section 2.4.2) and analysed by SDS-PAGE and Western blotting using monoclonal anti-c-Myc antibodies (section 2.4.5).

The results of this experiment (Figure 5.1) show, first of all, the production of the c-Myc-tagged Prp45p with a molecular mass of ~ 64-66 kDa (and even slower on SDS—10% PAA gels; see later in Figures 5.2 and 5.3), which is slightly higher than the calculated molecular weight of 59 kDa (42.5 kDa for Prp45p plus 16.5 kDa for the thirteen c-Myc epitopes). A previous study in our lab revealed the same decreased electrophoretic mobility of the recombinant His₆-Prp45p and a Protein A-tagged Prp45p on SDS-polyacrylamide gels indicating an apparent molecular weight
of 50-55 kDa instead of 42.5 kDa. The highly charged nature of this polypeptide could explain this discrepancy between the calculated molecular weight and its migration in gels.

Secondly, the Western blot showed that the two mutant proteins are quite stable at elevated temperature. Unlike the wild-type protein, the levels of the mutant proteins are slightly reduced in the samples taken at the last time points, 15 hours for YMM45-57 and 9 hours for YMM45-113 (Figure 5.1.A-lane 6 and B-lane 6). This is most likely due to the cessation of growth of the mutant strains by these late time points, which results in general proteolysis.
Figure 5.1 Western blot analysis of the Ts' mutant strains after the shift to 37°C. Strains YMM45, YMM45-57 (A.) and YMM45-113 (B.) were grown on YPDA liquid media at 23°C and 37°C, aliquots (3 OD_{600} units) were removed at specific time points and approximately 0.3 – 0.5 OD_{600} unit equivalents of cell extracts were analysed by SDS-PAGE. Protein extract prepared from wild-type strain (BMA38a) served as negative control. The gels were blotted onto PVDF membrane and probed with mouse monoclonal anti-c-Myc antibodies. The positions and the sizes of the marker proteins are indicated on the left of each gel by arrows.

Interestingly, the Prp45-113 protein seemed to migrate slower on this type of gel (Novex Minigel). To elucidate this migration behavior, the experiment was performed on another system: SDS-10% polyacrylamide gel electrophoresis (see section 2.4.4.2) of protein samples derived from crude extracts or splicing extracts were compared (Figure 5.2). Splicing extract was made from each strain of interest (YMM45, YMM45-57 and YMM45-113, grown on liquid YPDA at 23°C) as described in section 2.4.3 and the total protein concentration was estimated by Bradford Assay (section 2.4.1). Equivalent amounts of protein (170 μg) were electrophoresed.
Figure 5.2 Different electrophoretic mobilities of proteins expressed in \textit{prp45 Ts} mutants. Total protein prepared by TCA precipitation of crude extracts (lanes 1 – 4) or derived from splicing extracts (lanes 5 – 7) of the indicated strains were loaded onto a 10% polyacrylamide gel. The gel was run for about 7 hours at low voltage to allow a good separation of the protein species and then it was analyzed by immunoblotting using monoclonal anti-c-Myc antibodies. BMA38a protein extract (lane 1) served as a negative control. The position and sizes (in kDa) of marker proteins are indicated at the left. (*) represent the breakdown products of c-Myc-Prp45p as results of the proteolytic activity within the splicing extracts.

The results presented in Figure 5.2 strengthened what was determined previously, that both mutant proteins have a retarded migration behavior compared with the wild-type, with Prp45-57 being retarded less than Prp45-113 protein.
5.3 Analysis of the phosphorylation status of Prp45p

The previous experiment also revealed the existence of three bands corresponding to the Prp45 protein, possibly three putative isoforms generated by post-translational modifications. In 1998, Neubauer et al. purified and identified the spliceosome-associated proteins in mammals using gel filtration and affinity chromatography followed by two-dimensional gel electrophoresis. One of the spliceosomal proteins identified was the mammalian homologue of Prp45p, SKIP, which separated as three spots possibly corresponding to three different phosphorylation states. The extensive two-hybrid screens done in our lab that involved Prp45p (Albers et al., 2003), showed the interaction of this protein with protein kinase C protein, Pkc1p, suggesting possible phosphorylation events on Prp45p may occur. This is strengthened by the presence of 7 putative Protein kinase C phosphorylation sites and 7 putative Casein kinase II phosphorylation sites on Prp45p, as predicted by the PROSITE motif search programme (http://npsa-pbil.ibcp.fr/cgi-bin/pattern_prosite.pl).

Considering this, plus the extensive work done on SKIP as a factor involved in regulation of different transcription pathways - that is known to involve phosphorylation / dephosphorylation mechanisms - plus the results obtained in this study on the SDS-10% polyacrylamide gel electrophoresis, it was reasonable to examine the phosphorylation status of Prp45p. To achieve this, a co-immunoprecipitation of Prp45p-c-Myc fusion protein from YMM45 splicing extract was performed. The incubation of the splicing extract with the mouse anti-c-Myc antibodies was done in the presence of phosphatase inhibitors and protease inhibitors in order to maintain the phosphorylation state and the integrity of the protein and thus the multi-band pattern specific for phosphorylated protein. This step was followed by SDS-PAGE on 10% polyacrylamide gel and Western blotting with rabbit anti-c-Myc antibodies. As a control, the precipitate was digested with λ-protein phosphatase to remove any phosphate groups that Prp45p contains.
Figure 5.3 Analysis of the phosphorylation state of Prp45p. c-Myc-tagged Prp45p was immunoprecipitated from 50 μl of YMM45 splicing extract using mouse monoclonal anti-c-Myc antibodies. Half of the precipitate (P +) was treated with λ-protein phosphatase before being loaded onto 10% polyacrylamide gel (lane 4). This was run alongside the untreated precipitate (P -), an aliquot of the YMM45 splicing extract containing ~170 μg total protein (I – lane 1 and 5) and 3% of the total immunoprecipitation supernate (SN). An equivalent amount of protein derived from YMM45-57 splicing extract served also as control (lane 7). The samples were immunoblotted with rabbit polyclonal anti-c-Myc antibodies. On the left are indicated the positions of putative Prp45p isoforms as well as the sizes of the marker proteins. (*) represent breakdown products of Prp45p present in the splicing extracts.
This dephosphorylation treatment would determine a band shift in the migration of Prp45p, i.e. the retarded bands that correspond to the phosphorylated isoforms would disappear and more protein would migrate like the unphosphorylated form. In Figure 5.3 the results of the co-immunoprecipitation experiments are presented. The untreated Prp45p precipitate migrated as previously as three bands (lane 3). The two slowly migrating species, which probably correspond to phosphorylated isoforms of Prp45p, are very weak, maybe due to intrinsic phosphatase / protease activity in the splicing extract which is not completely abolished. This is confirmed by the absence of these species in the supernate and the presence of breakdown products. The λ-phosphatase treatment eliminated the slowest migrating band, while the intermediate one had a fuzzy appearance, maybe as a result of proteolytic activity.

Different modified protocols were used to demonstrate Prp45p phosphorylation, among which it is worth mentioning the use of different amounts and incubation times for λ-phosphatase, the use of Calf Intestinal Phosphatase or different immunoprecipitation buffers. Unfortunately, the results have not been more convincing. Thus it can not be concluded that, under these experimental procedures, Prp45p is phosphorylated and perhaps other protocols could be tried like two-dimensional gels or phosphorylation assays using [γ-32P] ATP, for which there was no time.

5.4 Prp45p mutant alleles and Prp46p interact in vitro

The mutations in regions A and B that confer the temperature-sensitive phenotype to prp45 alleles lie in the region R1 proved to interact in vitro with Prp46p (section 3.2). Therefore it was of interest to find out if these mutations affect the interaction with Prp46p and to achieve this, the same method was used as previously presented.

The prp45 Ts’ alleles containing the engineered mutations mutations in regions A and B were amplified from plasmids p57AB and p113AB (see section 4.3).
Figure 5.4 Analysis of the interaction between Prp46p and *prp45* Ts' mutant proteins.

A. $^{35}$S-labelled Prp45p, wild-type (lane 1) and R1 truncated form (lane 4) and Ts' mutant proteins, Prp45-57p and Prp45-113p (lanes 2 and 3) as well as the Prp45-113p truncated form R1 (lane 5) were *in vitro* synthesized using reticulocyte TNT extract (section 2.4.6). The TNT reaction was also performed with luciferase template (Luc) as a positive control or without DNA template (-DNA) as a negative control. Five microlitres from each reaction were mixed with 5 μl of 2X SDS-loading buffer and migrated onto a 4-12% PAA gel (Novex minigel, Novagen). The gel was dried and the radiolabelled proteins visualised by autoradiography.

B. HA2-Prp46p from yeast extract was mixed with $[^{35}]$S-methionine–labelled Prp45p, Prp45-57p or Prp45-113p and with anti-HA antibodies and incubated in a buffer containing 150 mM, 300 mM or 500 mM of NaCl (as described in section 2.4.7). The reactions were added to Protein A-Sepharose beads and incubated for 2 hours at 4°C. The beads, Precipitates, (lanes 1-3) were washed in buffer containing the same concentration of NaCl, then resuspended in 2X-SDS protein loading buffer and resolved on a 4-12% PAA Novex minigel. After electrophoresis, the gel was dried and the labelled proteins visualized by autoradiography. Samples without HA2-Prp46p-containing yeast extracts (lanes 4-6) were included as negative controls. To check for the stability of the proteins, supernates from each immunoprecipitation (1.6%) (lanes 7-12) were loaded alongside. The marker protein sizes and positions (in kDa) are indicated on the left.
A.

B.

Precipitates | Supernates

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The PCR products were used in TNT reactions (as described in section 2.4.6) with added $^{35}$S-methionine to get radiolabelled Prp45 proteins. For Prp45-113 mutant protein, an R1 region containing the N-terminal 218 amino acid residues was also synthesized. The results of the \textit{in vitro} transcription/translation reactions are presented in Figure 5.4.A. Note the different electrophoretic behaviour of the mutant proteins (lanes 2 and 3) compared with the wild-type (lane 1), which is supported by the differential migration of the truncated derivatives R1 (lanes 4 and 5). These results confirmed those obtained previously on immunoblots with anti-c-Myc antibodies (Figure 5.2), namely that the different migration pattern is due only to the substitutions generated in \textit{PRP45} ORF.

Co-immunoprecipitation experiments were performed with HA$_2$-Prp46p from yeast extract. In order to estimate the strength the \textit{in vitro} interaction, the experiment was performed at different salt concentration of the co-immunoprecipitation buffer ranging from 150 to 500 mM NaCl. The analysis was performed in parallel with the wild-type Prp45p.

As shown in Figure 5.4.B, HA$_2$:Prp46p co-precipitated full-length wild-type Prp45p even at 500 mM NaCl, which indicated a very strong interaction between the two proteins (Figure 5.4 B, upper panel). This supported the results obtained \textit{in vivo} by two–hybrid screens that identified a strong Prp45p-Prp46p interaction resistant to 20 mM triaminotriazole, 3-AT, a \textit{HIS3} inhibitor used to evaluate the strength of two-hybrid interactions.

Surprisingly, both Prp45-57p and Prp45-113p co-precipitated with Prp46p even at higher salt concentrations, like the wild-type, which means that the mutations they carry are not involved in this association and might affect the cell wellbeing through different interactions (Figure 5.4.B, middle and bottom panels).
5.5 Discussion

Following the construction of two temperature-sensitive mutants of \( PRP45 \), it was of interest to analyse how their mutations affect the stability, folding and interactors of the proteins they encoded.

The different migration behaviour on SDS-PAGE of Ts\(^+\) mutant proteins compared to the wild-type Prp45p is possibly due to the additional negative charges of these proteins. Data in the literature on HPV E7 protein from two different strains of human papillomavirus, HPV16 and HPV6, showed the same anomalous electrophoretic behaviour of these two proteins although the theoretical molecular mass is the same for both (Armstrong \textit{et al.}, 1993). The analysis of the two proteins showed that the different migration on SDS-PAGE was due to the presence of one aspartic acid residue in HPV16 E7 instead of one lysine as in HPV6 E7, this change being necessary and sufficient for the HPV16 E7 protein to have a slower electrophoretic mobility. As in the study presented here, in the \( prp45 \) Ts\(^+\) mutant proteins the extra glutamate residues in region \( A \), perhaps create a repulsion with the negatively-charged SDS, so there are less SDS bound than for the wild-type Prp45p. The overall charge is less negative for the mutant proteins and therefore, they will migrate more slowly towards the positively-charged electrode (anode) during electrophoresis. To support this theory, an extra experiment would be needed in which the protein samples are treated with a neutralising agent, for example, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) which converts the negatively charged carboxylates of amino-acid residues to neutral amides. It is also possible that the different negative charge of these proteins caused differences in the shapes of the proteins or SDS-protein complexes. To clarify this aspect, the experiment should be performed in the presence of a strong denaturing agent, like urea, which is known to destroy the secondary structure of the proteins.
The co-immunoprecipitation assay showed that mutations in regions \( A \) and \( B \) of the conserved SNW domain did not affect the interaction with Prp46p although they impaired the cell growth at elevated temperature. There are some explanations of this phenomenon. One could be that the mutations in regions \( A \) and \( B \) affect the interaction with different partners. Indeed, subsequent two-hybrid screens results (performed at Hybrigenics) narrowed down the minimum region of interaction with Prp46p within amino acids 55 and 98, approximately 30 amino acids upstream of region \( A \) (see Figure 3.1.A).

Another explanation is that the co-immunoprecipitation conditions might not alter the folding of the mutant proteins to the same extent as the \textit{in vivo} conditions, namely the growth at 37°C, might do. For this reason it would be worth analysing the interaction between the Ts' mutant proteins and Prp46p \textit{in vivo}, by two-hybrid assay. Both mutants could be cloned in bait plasmids and the interaction with Prp46p produced from a prey plasmid should be assessed by growing the transformed yeast cells at 37°C and on different concentrations of 3AT.

Although there was much data that suggested a phosphorylated state of Prp45p, the experiments performed in this direction have not given a conclusive result. However, it is possible that other post-translational modifications could affect Prp45p. In this respect, the PROSITE motif search of Prp45p predicts the existence of three putative N-glycosylation sites on three asparagine residues, two of them being situated upstream of region \( A \) (position 107) and of region \( B \) (at position 194).
Chapter 6  Pre-mRNA Processing in prp45 Ts\textsuperscript{-} Mutants
6.1 Introduction

Previous work has demonstrated the role of Prp45p in pre-mRNA splicing in vivo and in vitro (Albers et al., 2003). The analyses were done on a galactose-regulated PRP45 strain, in which the expression of PRP45 and consequently its function could be switched off by the addition of glucose. Northern blotting and primer extension analysis showed that pre-mRNA started to accumulate after 8 hours in glucose-based medium, indicating Prp45p requirement for splicing in vivo. Following metabolic depletion of Prp45p, cell extracts could not support the formation of splicing intermediates and products from actin pre-mRNA and, as shown in Chapter 3, the formation of active spliceosome was inhibited. Further work involving co-immunoprecipitation of RNA species by proteinA: Prp45p showed the association of Prp45p with the splicing machinery in both steps of splicing.

Considering these data, it was of interest to determine the involvement of newly identified prp45 Ts' mutants in pre-mRNA processing and further, at what particular catalytic stage these mutants are deficient. The analyses presented in this chapter include not only classic experiments (Northern blotting, in vitro splicing assay) but also reporter gene assays and newly developed microarray assays.

6.2 Effect of prp45 Ts' mutants on pre-mRNA splicing in vivo

RNA from prp45 mutant strains YMM45-57 and YMM45-113 grown at non-permissive temperature was analysed for the accumulation of pre-mRNA, indicative of a splicing defect in vivo. Both strains were grown at 23°C until mid-logarithmic phase and then shifted to 37°C for 1, 2 and 4 hours. Total RNA was isolated and analysed by agarose gel electrophoresis and Northern blotting with a probe to exon 2 of the ACT1 gene. PRP45 strain, YMM45, was used as a negative control.
Figure 6.1. The *prp45* temperature-sensitive yeast strains do not accumulate actin pre-mRNA *in vivo*. Northern analysis of the total RNA extracted from WT (YMM45) (lanes 1-4), YMM45-57 (lanes 5-8), YMM45-113 (lanes 9-12), and *prp16-2* (lanes 16 and 17) grown at 37°C in YPDA liquid media for the specified time. YMA45/2 was pre-grown at 30°C in galactose-based media to mid-logarithmic phase before being transferred to glucose (YPDA) for 6 and 12 hours (lanes 13-15). Time 0 represents the point when the shift was done from permissive to non-permissive conditions. Ten micrograms of RNA was run on a 2% (w/v) agarose gel and then blotted onto Hybond-N membrane (Amersham). The blot was hybridised with a radiolabelled oligo complementary to exon 2 of the *ACT1* gene. The position of the *ACT1* pre-mRNA and mRNA are indicated. The same filter was stripped and reprobed with a *PGK1* gene probe as a control for loading (bottom lanes).
Two splicing-defective strains were positive controls: 1) YMA45/2 strain grown in glucose-based medium for 6 and 12 hours to allow the full repression of \textit{PRP45} expression; 2) \textit{prpl6-2} temperature-sensitive strain grown at 37°C for 2 hours. Prp16p is a member of the DEAH-box family and is an ATP-dependent helicase required for the second step of splicing – cleavage at the 3’ splice site and exon ligation (Schwer and Guthrie, 1991).

Figure 6.1 shows that no pre-mRNA was detected in either YMM45-57 or YMM45-113, nor a decrease in the level of mRNA (lanes 6-8 and 10-12). This is not the case for YMA45/2 that, after 12 hours in glucose-based medium, accumulated precursor actin RNA while the level of spliced mRNA decreased (lane 15). This is a known behaviour of the strains defective in splicing that have rapidly degraded pre-mRNA and mRNA species in the nucleus (Bousquet-Antonelli et al., 2000). \textit{prpl6-2} showed no pre-mRNA accumulation but the level of mRNA decreased, as expected for a defect in the second step of splicing while the amount of the loading control, \textit{PGKI} mRNA, (bottom panel, lane 17) remained unaffected.

6.3 \textit{\textbeta}-Galactosidase Assay

To extend this analysis, a more sensitive method was employed that detects reductions in the level of splicing not visible using Northern blotting. The assay also detects transport of the unspliced pre-mRNA from the nucleus to the cytoplasm.

Briefly, the \textit{\textbeta}-galactosidase assay involves three \textit{lacZ}-based reporter genes placed under the control of a galactose-inducible promoter (Figure 6.2.A). Plasmid pLGSD5 contains the \textit{P\textsubscript{GAL-CYC1-lacZ}} hybrid gene and allows detection of defects in transcription / translation of the \textit{\textbeta}-galactosidase gene. Plasmid pJC51 contains a synthetic sub-optimal \textit{RP51A} intron flanked by short exons. The intron has to be spliced out for the production of \textit{\textbeta}-galactosidase as the \textit{\textbeta}-gal coding sequence is out-of-frame with \textit{CYC1} AUG in the unspliced RNA.
Figure 6.2. Effect of prp45 Tn- mutants on pre-mRNA export, pre-mRNA splicing and transcription/translation.

A. Constructs used in β-galactosidase assay. The star symbol represents frame-shift mutation. On the right is shown their ability to express β-galactosidase. B. YMM45 (wild type PRP45), YMM45-57 (prp45-57), YMM45-113 (prp45-113) and prp8-1 strains were grown in liquid GGL-U media. Upon reaching an OD_{600} of 0.5, β-galactosidase expression was induced for 2 hours at non-permissive temperature (37°C) by 2% (v/v) galactose. Aliquots of 1 ml were removed from each culture, the cells were broken and the β-galactosidase activity was estimated using a colorimetric assay as describe in section 2.2.9. Four independent transformants were analysed in duplicate and the results are expressed as means of β-galactosidase activity ± SD.
A.

\[
\begin{align*}
\text{pLGSD5} & \quad \text{mRNA} \quad \text{GAL} \quad \text{ATG} \quad \text{Lac Z} \\
\text{pre-mRNA} & \quad \text{GAL} \quad \text{ATG} \quad \text{intron} \quad \text{Lac Z} \\
\text{pJC51} & \quad \text{mRNA} \quad \text{GAL} \quad \text{ATG} \quad \text{Lac Z} \\
\text{pre-mRNA} & \quad \text{GAL} \quad \text{ATG} \quad \text{intron} \quad \text{Lac Z} \\
\text{pJC1} & \quad \text{mRNA} \quad \text{GAL} \quad \text{ATG} \quad \text{Lac Z}
\end{align*}
\]

\[\beta\text{-Gal Activity}\]

yes

no

yes

no

B.

\[\beta\text{-GALACOSIDASE ASSAY}\]

\[\text{ACTIVITY (Units)}\]

\[\text{REPORTER PLASMID}\]

\[\text{pJC1} \quad \text{pJC51} \quad \text{pLGSD5}\]
The last construct, pJC1, resembles pJC51 except that the β-galactosidase ORF is in frame with the CYCI AUG only for the unspliced pre-mRNA, so that β-galactosidase is produced only when the intron is not removed.

This plasmid is a tool for detecting pre-mRNA export into the cytoplasm. prp mutants that are deficient in the early steps of spliceosome assembly may permit the exit of unspliced pre-mRNA from the nucleus and, subsequently, their translation into protein in the cytoplasm. This method also gives information on the putative effect that these Ts' mutants have on transcription / translation. This is of particular interest, as certain homologues of Prp45p like human SKIP and D. melanogaster NcoA-62, have been shown to have roles as transcription co-regulators.

The three constructs were transformed into the YMM45-57, YMM45-113 and YMM45 (wild-type) strains and the splicing-deficient strain prp8-1. The splicing factor Prp8p is a U5 snRNP specific protein required for splicing in vivo and in vitro (Larkin et al., 1983, Lustig et al., 1986). The transformants were grown in glycerol / lactate-containing medium at 23°C and β-galactosidase activity was measured 2 hours after the addition of galactose and a shift to 37°C (see section 2.2.9). The results are presented in Figure 6.2.13.

The pLGSD5-transformed cells gave rise to significant β-galactosidase activity and no major difference could be seen between the prp45 Ts' mutants and the wild-type cells. These results mean that the substitutions in regions A and B of Prp45p did not affect the transcriptional or translational activity, at least not in the case of this reporter gene. The level of β-galactosidase in prp8-1 mutant is much lower, around 42% of level of the wild-type. This indicated a possible role Prp8p might have in transcription. Links between transcription and splicing are known (for reviews, see Proudfoot et al., 2002; Hirose and Manley, 2000).

As expected for the splicing reporter assay (pJC51), the prp8 mutant strain displayed very low β-galactosidase activity, about thirty-fold less than YMM45. The level of the enzyme activity in YMM45-57 and YMM45-113 after the galactose induction was 70% and 52%, respectively, compared to wild-type, indicating a mild splicing defect in vivo, with one stronger than the other.
With pJC1, the \textit{prp8-1} cells showed a 5-fold increase in translation compared to wild-type, and about 15% of the \(\beta\)-galactosidase activity of pLGSD5 transformed in the same strain. The latter result was similar to that reported previously by Legrain and Rosbash, 1989, for the same mutant, then known as \textit{rna8-1}, while, analysing strains defective in the early steps of spliceosome assembly (\textit{rna6}), the relative \(\beta\)-galactosidase activity was about 80%. As for the \textit{prp45} mutants, the \(\beta\)-galactosidase activities as percentage of pLGSD5 activity were: 5.6% and 2.7%. Therefore this experiment re-confirmed the results of Lustig \textit{et al.}, 1986 and those reported in Chapter 2, that both Prp8p and Prp45p are dispensable for the early steps of spliceosome assembly.

The 50% reduction in splicing activity indicating an \textit{in vivo} splicing defect stronger than for YMM45-57 (with 30% reduction) was a good reason to focus only on YMM45-113 mutant strain for further analysis. In the following section this mutant was investigated to determine at which step the splicing inhibition occurred.

### 6.4 Effect of \textit{prp45} Ts' mutants on pre-mRNA splicing \textit{in vitro}

To analyse pre-mRNA splicing \textit{in vitro}, splicing extract was prepared from strain YMM45-113 (Ts') grown continuously at 23°C and \textit{in vitro} splicing reaction was performed as described in section 2.3.3.6. In order to establish the optimal conditions to detect a splicing defect \textit{in vitro}, the reactions were incubated at different temperatures, i.e. at 23°C (25 min), 28°C (15 min), 30°C (15 min), 32°C (12 min) and 34°C (12 min) (Figure 6.3). To avoid some errors that could arise due to elevated temperature that is known to activate nucleases; therefore an extract derived from wild-type \textit{PRP45} strain YMM45 was prepared and analysed alongside. To eliminate a potential defect caused by the limiting amount of splicing factors, the protein concentration of both extracts was measured by Bradford Assay and the same protein amount was used in the assay.
Figure 6.3 *In vitro* splicing assays of *prp45* mutant strain performed at different temperatures.

YMM45 (WT) and YMM45-113 (113) yeast strains were grown in YPDA at 23°C. Upon reaching an OD$_{600}$ of 0.7, splicing extracts were prepared. Five microliters of splicing extract plus splicing buffer, ATP and radiolabelled actin pre-mRNA (substrate, lane 2) were mixed and the splicing reactions were incubated at the indicated temperature for different times. The splicing reactions were stopped, deproteinised and the RNA species were extracted with phenol/chlorophorm/isoamylic alcohol (P/C/I) and then ethanol-precipitated before being loaded on a 7% polyacrylamide gel. A radiolabelled DNA marker (*MspI* digested pBR322) was loaded alongside (lane 1). The splicing species that result from the first and the second step of splicing are indicated on the right side.
It can be noticed from Figure 6.3 that, according to the amount of mRNA formed, the two extracts started to display different activities upon heat treatment at 28°C. The 15 minutes incubation of prp45-113 at 30°C (lanes 7 and 8) caused a stringent reduction in the splicing intermediates and products, especially second step ones, lariat intron and mRNA. It is clear from the results in lanes 9 and 10 that the incubation above 32°C triggered the action of nucleases in both wild-type and mutant (a smearing appearance of RNA species).

Although the block was not complete (i.e. the production of second-step splicing intermediates was not abolished) these results indicated that the prp45 Ts mutant displayed a mild splicing defect in vitro, apparently affecting the second step of splicing more than the first. Other heat-inactivation conditions were applied to get a stronger in vitro splicing defect for the prp45 mutant. Unfortunately they did not lead to a more conclusive result. One example is the heat-treatment of both extracts at 32°C in the absence of ATP and pre-mRNA substrate followed by their addition and incubation at 15°C for 1 hour, or performing the splicing reaction at 37°C for 20 minutes. The latter experiment caused complete degradation of pre-mRNA species.

So far the 30°C incubation of splicing reactions seemed to get the most obvious defect for prp45 strain, and to confirm this finding, a time course was done. Splicing reactions were set up as previously and incubated at 30°C for 1.5, 5, 10 and 15 minutes. The results are depicted in Figure 6.4. Wild-type extract produced intermediates of the splicing reaction within 1.5 min and mature RNA formation, detected after 5 minutes exponentially accumulated up to 15 minutes. In contrast, extract from prp45 cells displayed a slower kinetics: the splicing intermediates started to accumulate after 5 min. (lane 7). The 15 minutes incubation limited the splicing of the actin pre-mRNA to less than 7% of that observed for the wild-type extract, as calculated for mRNA species by PhosphorImager quantification (lanes 5 and 9 and data not shown).
Figure 6.4 Time-course of the \textit{in vitro} splicing of the actin pre-mRNA performed at 30°C.

Five microliters of splicing extracts from wild-type and YMM45-113 strains prepared from cells grown at the permissive temperature of 23°C were mixed with splicing buffer, ATP and radiolabelled actin pre-mRNA. The reactions were incubated at 30°C for the indicated periods of time and then were P/C/I extracted and ethanol precipitated. The precipitated RNA was fractionated on a 7% denaturing polyacrylamide gel and the results were visualised by autoradiography. RNA species are cartooned on the right. In the first lane, a radiolabelled size marker, \textit{MspI}-digested pBR322 was loaded.
Taking together, the results presented in this section were compatible with those from the β-galactosidase assay, namely the prp45 Ts' mutant had a mild splicing defect. At that moment a question was posed: if the splicing defect was mild then what determined, at a molecular level, the strong growth defect of prp45 mutants at 37°C? A possible explanation could be that the mutant protein affects the splicing of other transcripts more severely; therefore a more global assay for pre-mRNA splicing analysis was employed using microarray technology and is presented in the following section.

6.5 YMM45-57 and YMM45-113 accumulate intron-encoded snoRNA species at non-permissive temperature

6.5.1 Microarray analysis of YMM45-57 and YMM45-113

Contrary to the traditional use of a small number of reporter genes to characterize a splicing defect, the microarray method analyses all yeast intron-containing transcripts.

The method consists of two parts: 1) designing the microarray (biochip), that was achieved by our colleague, David Barrass; 2) generating the fluorescent labeled cDNA, hybridizing it to the microarray and analyzing the data (see section 2.3.3.4).

1) The microarray contains four probes engineered to recognize and bind specifically sequences from each yeast intron-containing transcript: one for the 5’-splice site (V), one for the intron (I), one for the 3’ exon (E) and one for the mature junction that forms between linked exons. All these probes are transferred from a micro-well plate onto a glass surface by a printhead containing microspotting pins. To avoid errors, each set of probes is transferred in triplicate; therefore there will be three sets of results for each intron-containing transcript from each microarray.
Figure 6.5 Microarray design and pre-mRNA splicing pathway for normal transcript (A.) and snoRNA-intron containing transcripts (B.)

The microarray contains four probes for each intron-containing transcript: intron probes (red) detect pre-mRNA and lariats; 5'-splice site (pink) detect pre-mRNA; mature junction (green) probes detect mRNA and exon 2 probes (orange) detect both pre-mRNA, lariat intermediate and mRNA. The arrays for snoRNA-intron containing transcripts have an additional probe corresponding to the mature snoRNA (purple) and detect snoRNA-containing species.
2) Yeast total RNA was extracted from YMM45 and YMM45-57 or YMM45-113 strains grown at 37°C for a period of time determined from the growth curves (Figure 4.3).

A reverse transcription reaction was set up with the RNA and two fluorescent-labeled dTTP, namely Cy3 for the wild-type RNA and Cy5 for the mutant RNA were included (forward labeling reaction). They were incorporated during extension into the DNA strand so that the resulting cDNA molecules would absorb and emit light at distinct and separable wavelengths. In order to minimize differences that could affect the final results, care was taken to ensure that the genetic background of the strains analyzed was the same: YMM45, YMM45-57 and YMM45-113 are all derived from BMA38a and the only difference between them is the mutations in the PRP45 ORF. Moreover, the growth conditions (the media, temperature, shaking speed), final OD600 at which the cells were collected and the RNA extractions and manipulations were similar for the wild-type and mutant strains. Finally, any variance that could result from different dye incorporation or fluorescence was ruled out by swapping the dyes between the wild-type and mutant cDNA (reverse labeling reaction) for all samples.

The two fluorescent cDNA pools corresponding to the wild-type and to the mutant strain were purified, mixed and hybridized to the microarray overnight, at 47°C. After the hybridization, the unbound material was removed by successive washings and the fluorescent DNA bound to each probe on the biochip was visualized by fluorescence detection. The glass slide was subjected to white light excitation and the light emitted from the two fluorescent samples at each location was converted to a digital output, quantified and interpreted. More specifically, in an ideal situation, the light emitted by the wild-type cDNA (Cy 3) would hybridize differentially to the four probes of a transcript resulting in a certain pattern of intensities from spots, namely signals from M (green) and E (orange) probes (Figure 6.4). If the mutant does not affect splicing, i.e. there are no intron species accumulating, the pattern of intensities would be approximately the same, between the mutant and the wild-type.
Figure 6.6 Scatter plots of probe intensities for prp45 Ts\textsuperscript{+} mutants at different time points after shift to non-permissive temperature (37°C).

YMM45-57 and YMM45-113 temperature sensitive strains were grown in YPDA at 23°C until mid-log phase and shifted to 37°C. At the indicated time points, total RNA was prepared and used in microarray analysis. The intensities of probes were plotted on log\textsubscript{10} graphs where x axis represents the wild-type (Cy3) and y axis represents the mutant (Cy5). The results presented are for the direct labelling reaction. The intensities of the spots are coloured as in Figure 6.5: red for intron probes, pink for 5' splice-site probes, green for mature junction and orange for exon 2 probes. The purple spots correspond to the intron encoded-snoRNA probes and are different from the intron probes of the same transcript. The ellipses delimit the spots characteristic for a certain species and are colour-coded as before.
Conversely, if the mutant is fully splicing-defective, the Cy5 signals are detected for I (red), V (pink) and E (orange) probes. The pattern that results after the combination of the signals would be different than in wild-type and is indicative of a splicing defect. In reality it would be a gradient of intensities in both cases.

The probe intensities are plotted on a graph: YMM45 (Cy3 on x axis) versus YMM45-57 or YMM45-113 (Cy5 on y axis) for different time points in accordance with the growth rates (Figure 4.9.B). Figure 6.5 show the scatter-plots for the two mutant strains at permissive (0h) and non-permissive temperature. It can be seen that there is no widespread accumulation of the intron probes (red spots) or 5'splice site probes (pink spots) in the mutant strains relative to the wild-type even after longer exposure at the non-permissive temperature. The Cy5/Cy3 ratios (representing the correlation between the mutant and wild-type) remained unaffected for the other two probes, mature junction (M) and exon (E) for almost all genes. This indicates that there is no detectable splicing defect caused by the two prp45 mutants at elevated temperature and the results validated what was found in the previous sections of this chapter. The reverse labeling reactions confirmed these results were accurate.

However, two interesting things were conspicuous in these scatter-plots: firstly, the specific accumulation in both mutants of intron probes of transcripts like YMR116C, RPL7A and RPL7B - in the case of the YMM45-113 strain the red spots are obvious even at 23°C. Transcript, YMR116C (ASC1, BEL1) encodes for a WD-repeat protein (G-beta like protein) and is believed to interact with the translational machinery. RPL7A (YGL076C) encodes for the ribosomal protein L7A and RPL7B (YPL198W) encodes for the ribosomal protein L7B. Null mutants of either RPL7A or RPL7B are viable but disruption of both is lethal (submitted information to SGD).

Secondly, the plots showed the accumulation of the small nucleolar RNA specific signals. They are colored in purple in order to distinguish them from the other intron probes (see Figure 6.4.B). This effect can be seen at 23°C for YMM45-113 and then in both mutants at later time-points. Small nucleolar RNAs (snoRNAs) represent an abundant and diverse class of non-coding RNAs that play a major role in posttranscriptional covalent modifications (2'-O-methylation, pseudouridylation and cleavage) of different groups of RNA (rRNA, tRNA and snRNA) (Allmang et al., 1999; for review see Kiss, 2002). In yeast, the majority of snoRNA are derived from
independently transcribed units either mono- or poly-cistronic, possessing their own promoter and transcribed by RNA Pol II (like U3, U13, U14) or RNA Pol III (like MRP). Apart from these, seven snoRNAs (U18, U24, snR38, snR44, snR39, snR54 and snR59) are encoded in introns of protein-coding genes.

In the scatter-plots illustrated in Figures 6.5 all the intron-encoded snoRNAs were identified; YMR116C is the host gene for snR24 (U24), RPL7A accommodates snR39 while RPL7B is the host for snR59. Interestingly, there is no accumulation of snR17A (U3 snoRNA) either intron probe or 5' splice site probe. The non-intron encoded snoRNA, snR10, that served as control as well as snRNAs (U1, U2, U4, U5 and U6) were not affected (their signals were on the median). Microarray analysis performed in our lab for various Ts' mutants showed that this defect depends on the function of the defective protein within the cell. For example, the exosome Ts' mutant rrp6 strongly accumulated snoRNA-containing species after 30 minutes at 37°C, the second step mutant prp16-2 showed a similar pattern to prp45 mutants while the splicing mutant prp8-1 accumulated intron probes but not snoRNA containing ones (David Barrass, personal communication).

An important step of microarray analysis is generating dendrogram clusters that identify the relationship between different mutants with respect to their effects on different probes, in this case intron probes (Figure 6.6, left panel) and 5'-splice site probes (Figure 6.6, right panel) of all the microarray transcripts. So far the mutants analyzed in our lab clustered into two major groups: one, located in the top left side formed by the exosome mutants (rrp6, ski6) and a very large group in the middle of the clusters consisting of prp mutants prp2, prp8, prp16, prp18 (David Barrass and Jean Beggs, unpublished data). Both prp45 mutants are clustering with the latter group. Interestingly, according to the intron index clustering, prp45-57 seemed to behave similarly with the prp16-2. However the number of transcripts whose splicing is affected in prp45 mutants is lower than for the other splicing mutants and further analysis is needed to identify the nature of these transcripts.
Figure 6.7 Dendrograms clustered by two indexes: the intron accumulation and 5'splice-site accumulation, 5'SS. 

prp45 mutants and other temperature-sensitive mutants analysed in our lab were clustered using the Average Linkage (UPGMA) cluster method according to the similarities in the effects they have on the intron accumulation indices (left panel), and 5'splice-site accumulation indices (right panel). The resulting dendrograms align the analysed mutants on the horizontal axis, and on the vertical axis all the intron containing transcripts. The corresponding red boxes represent the extent of index accumulation in the mutants compared with the wild-type.
6.5.2 Northern blot of YMM45-57 and YMM45-113 confirmed the microarray analysis

There are two ways in which intron-containing snoRNAs are produced. One is the splicing-dependent pathway that involves the formation of the snoRNA-containing intron lariat. Following the splicing reaction, the lariat is linearised by a debranching enzyme (Prp26p/Dbr1) and it is processed into a mature snoRNA by trimming at the ends by nuclear 5'→3' exonuclease Rat1 and components of the nuclear exosome (3'→5' exonucleases) (Petfalski et al., 1998, Ooi et al., 1998). An alternative, minor pathway is the splicing-independent one involving the activity of some endonucleases like Rnt1 on the snoRNA-containing pre-mRNA and then the 5' and 3' trimming at the ends (Giorgi et al., 2001).

In order to confirm the results obtained in the microarray experiments, Northern Blotting analyses were performed. The filter shown in Figure 6.1 was successively hybridized with oligonucleotides complementary to three intron-containing snoRNA genes, YMR116C (the host gene for U24), EFB1 (the host gene snR18) and TEF4 (the host gene for snR38) that constantly accumulate snoRNA signals in the prp45 mutants (Figure 6.7, top panel).

Under non-permissive conditions, GAL:PRP45 and prp16-2 strains (lanes 13-17) showed, as for ACT1 gene, accumulation of very low amounts of pre-mRNA and loss of the mRNA as well as the accumulation of 3'-truncated intermediate A (blots 1a, 2b, 2c, 3a) and of 5'-truncated intermediate B (blot 3a). Primer extension analysis in a previous study (Bousquet-Antonelli et al., 2000) identified the nature of these species: A extends to the 5'end of the transcript and is the result of a defective 5'→3' degradation pathway (Rat1 exonuclease defect), while B extends to the 3' end of the transcript and accumulated in the absence of 3'→5' exonucleases (exosome components). The same work showed data compatible with one presented here, i.e. the accumulation of A intermediates is stronger than B-forms (detectable here only in case of TEF4) due to a 3'→5' degradation more active than 5'→3'.
Figure 6.8 Analysis of pre-mRNA processing of the snoRNA-intron encoding genes YMR116C, EFB1 and TEF4.

In the upper panel it an intron-encoding snoRNA gene and the truncated intermediates A and B generated by nuclear pre-mRNA degradation are schematically illustrated. Strains were grown in non-permissive conditions for the indicated times and total RNA was prepared as described for Figure 6.1. Ten micrograms of RNA was fractionated either on a 2% (v/v) agarose gel (panels 1a, 1b, 2a, 2b, 2c, 3a) or on 6% denaturing polyacrylamide gel (panels 1c, 2d and 3b) to visualise the low molecular weight RNA species. The gels were hybridised with the oligo probes shown in brackets and their annealing position is illustrated in the upper panel. The names of the genes followed by the name of the intron-encoded snoRNA are on the left. The names and the positions of the RNA species generated by pre-mRNA splicing or degradation are indicated on the right.
In contrast with the *ACT1* gene, at 37°C the *prp45* Ts' mutants (Figure 6.7, lanes 5-12) had reduced levels of mRNA (panels 1a, 1b, 2a, 2b) and also accumulated A-forms of all tested snoRNA containing transcripts even at permissive temperatures, especially in the case of *prp45-113* which is a tighter Ts' mutant. The result is in agreement with the microarray data that showed that snoRNA-containing species are accumulating in this mutant even at permissive temperature (Figure 6.5, upper panel).

It was also observed that, when probing with primers annealing to exon 2, 423 (blot 1b) or 484 (blot 2a), the *prpl6-2* strain strongly accumulated one species smaller than the corresponding pre-mRNA. This also appeared (but not so obvious) for *prp45* mutants at elevated temperatures and after 6 hours of metabolic depletion of Prp45p in GAL-regulated strain. This specific band did not appear with hybridization against exon 1. The position of the band compared to the molecular markers run alongside (High Molecular Weight RNA Marker, Helena Biosciences) suggested that this species might correspond to the lariat-intron exon 2 intermediate that normally accumulates for step 2 splicing mutants. To validate this finding, and to rule out that this was a result of cross-hybridization, the filter was probed by an oligo against mature U18 (panel 2c).

Alternatively, the RNA samples were run on a 6% polyacrylamide gel that separates low molecular weight RNA species and blotted with oligonucleotides corresponding to mature U18 (205 – panel 2d) or mature U24 (214 – panel 1c). In *prpl6-2* cells (lane 17), upon probing for U18, there were two slower migrating bands besides mature snoRNA, the slowest one corresponding to the lariat intron-exon 2. Although the size of *EFBI* pre-mRNA is 81 nucleotides larger than the corresponding lariat intron-exon 2 (987 nt versus 906 nt), the latter one, being circular, will be retarded. This migration pattern is common to RNA species fractionated on polyacrylamide gels. As for *YMR116C*, one single band assumed to correspond to lariat intron-exon2 (625 nt) accumulates in *prpl6-2* mutant. The pre-mRNA species are longer (1233 nt) and could not be resolved. Indeed, *prp45* Ts' mutants seemed to modestly accumulate lariat intron-exon 2 intermediate at non-
Chapter 6: Pre-mRNA Processing in prp45 Ts' Mutants

permissive temperature (1b, 1c, 2a, 2d - lanes 6-8 and 10-12). This supports the in vitro splicing assay results showing that prp45-113 mutant (section 6.2) caused a mild step 2 splicing defect (Figure 6.3). In addition to this, analysis of Prp45p-depleted cells showed that, after 6 hours in glucose, EFBI and YMR116C lariat intron-exon 2 intermediates could be detected as a smearing band due perhaps to degradation (panels 1c and 2d, lane 14) while EFBI pre-mRNA was increasingly accumulating (blot 2d).

In all the strains analyzed, the mature snoRNAs formation was little affected by the mutant proteins (panels 1c, 2e, 3c). Processing towards a mature U18 or snR38 is done by the alternative endonucleolytic cleavage of the intron by Rnt1 endonuclease (Giorgi et al., 2001) or, as for U24 by exonucleolytic activity on the debranched intron lariats (Petfalski et al., 1998; Ooi et al., 1998; Allmang et al., 1999).

It can be concluded that the prp45 mutants presented the characteristics of splicing mutants defective in the second step of splicing. Accumulation of snoRNA-truncated intermediates suggested that unspliced pre-mRNA species are present but cannot be identified due to the rapid degradation by the 5'→3' and 3'→5' nuclear exonucleases (Bousquet-Antonelli et al., 2000).

6.6 Discussion

In this chapter the effects of prp45 Ts' mutants on pre-mRNA splicing and transcription were investigated.

The lack of a strong splicing defect in the prp45 temperature-sensitive strains suggests that the growth arrest these cells exhibit at elevated temperature may not result directly from a defect in pre-mRNA splicing. It is therefore possible that these mutant proteins are involved in other cellular processes, which are disrupted under the restrictive conditions. One process is transcription and the Prp45p role as a transcriptional co-regulator in other organisms, has long been discussed (Zhang et al., 2001, Leong et al., 2001, Zhou et al., 2002, Barry et al., 2003). However, the
reporter gene expression assay employed here did not support such a role, at least for
the prp45 mutants analyzed here.

On the other hand, although it was demonstrated that Prp45p is a general
splicing factor (Albers et al., 2003), it is possible that these mutant proteins are
involved in the splicing of a subset of transcripts with specific features. To clarify
this, a microarray analysis was employed and the results confirmed by Northern
blottings.

The microarray clustering data for 5'splice site probes and intron probes
showed similarities between prp45 mutants and other splicing factors in processing
different transcripts. However, the number of the transcripts affected is smaller than
for prp8 or prpl6 mutants and further statistical analysis of the data is required in
order to identify these transcripts.

A key question is why the truncated forms of snoRNA-containing genes
accumulated as a result of a splicing defect. This finding is supported by the
experiments on different splicing mutants like prp45 and prpl6-2 in this chapter, and
of prp2-1 in the study of Bousquet-Antonelli et al. (2000). The degradation
intermediates A and B resemble those accumulating for Rat1/Xrn1 mutants defective
5'→3' exonuclease activity and the exosome mutants defective in 3'→5'
exonucleolitic activity, and a question arises: why are these truncated forms
accumulating in splicing-defective strains? One answer could be that, by the time
these splicing factors are entering the spliceosome machinery, the pre-mRNA is 5'
capped and associated with different splicing factors that hinder the access of
exonucleases to 5' or 3' ends and, considering that these two events, pre-mRNA
splicing and nuclear pre-mRNA decay, are in competition (Bousquet-Antonelli et al.,
2000), the exonucleaseolytic degradation occurs with different rates.

Given that prp45 mutant strains appear to show a partial second-step splicing
defect affecting the snoRNA intron-containing transcripts and the ACT1 gene in in
vitro splicing assays, it is possible that Prp45p is involved in regulation at this stage.
To date, different studies showed that Prp45p is a member of U5 snRNP/Prp19 joint
protein complex required for the formation of the active spliceosome and involved in
both catalytic steps (Makarov et al., 2002, Ohi et al., 2002). Some proteins found in
this complex are involved in second-step splicing like Prp22p, Slu7p, Prp17p and
Prp8p (Schwer and Gross, 1998; Umen et al., 1995) but whether or not they directly interact with Prp45p is not known. So far, two-hybrid screen analysis (Albers et al., 2003) has suggested an interaction in vivo between Prp22p and Prp45p, which, given the fact that it is uni-directional, it has been assumed to be indirect. Considering this result, it is possible that Prp45p could regulate the second step of splicing through an interaction with Prp22p. As Prp22p is required for the splicing of transcripts with the distance between the branchpoint and the 3’splice site, [BP-3’SS], larger than 21 nucleotides (Schwer and Gross, 1998), one could assume that this interaction with Prp45p might occur only for such transcripts and that this interaction is affected in prp45 mutant strains. This is not the case for snoRNA-containing genes (for whom the second step splicing defect was observed) whose [BP-3’SS] are variable, from 19 nucleotides as in YMR116C/snR24 to 35 nucleotides as in EFB1/snR18. This is also improbable taking into account the fact that Prp45p is required to splice transcripts with [BP-3’SS] as small as 7 nucleotides (Albers et al., 2003). All these observations could imply that Prp45p might affect the second step of splicing for different transcripts through different pathways and further work would be needed to demonstrate them.

Overall, given the abundance of snoRNA-containing species within a cell and their stability due to association with many proteins, the use of snoRNA-intron encoding probes proved to be an important tool to detect pre-mRNA splicing defects in vivo.
Chapter 7: Final Discussion and Future Work

7.1 Introduction

Prp45p was isolated as a main interactor of Prp22p in a two-hybrid screen and subsequent analyses showed that it is a bona fide splicing factor required for nuclear pre-mRNA splicing both in vivo and in vitro (Albers et al., 2003). Co-immunoprecipitation experiments found Prp45p associated with the spliceosome before the first catalytic step and until the end of the splicing process when it leaves with the lariat intron. This study shows that this association occurs before the formation of the active spliceosome (complex C) but post complex B formation (inactive spliceosome) (Figure 1.3), most probably after U1 and U4 snRNAs have already left the spliceosome. Several lines of evidence are supporting this finding. Firstly the co-immunoprecipitation of spliceosome snRNA with Prp45p that showed its association with U2, U5, and U6 snRNAs, but not with U1 and U4 snRNAs (Albers et al., 2003). Secondly, the latest analyses demonstrated Prp45p to be a member of a large protein complex, Cwc/Ntc/35S complex, identified both in yeast and in human (Ohi et al., 2002; Chen et al., 2002; Makarov et al., 2002). These complexes were demonstrated to enter the spliceosome just after U1 and U4 snRNA dissociation and before the first catalytic step. These complexes include other proteins like Cef1p, Prp46p, Isy1p, Syf1p, Syf2p, Syf3p that were shown to interact with each other in two-hybrid screens and by co-immunoprecipitation analyses (Albers et al., 2003; Dix et al.; 1999; Ben-Yehuda et al., 2000). The data presented here confirmed the Prp45p-Prp46p interaction found in two-hybrid screens and in addition to this, mapped the region of interaction within the N-terminal domain of Prp45p.

In order to get more insights into the function/structure relationship of Prp45p, random mutagenesis of PRP45 gene was employed. Contrary to the theory that PRP45 could not render temperature-sensitive mutations (Harris et al., 1992), two Ts’ mutants were obtained, prp45-57 and prp45-113, and subjected to further analyses. Sequencing analyses revealed that the mutations are mainly located into
two discrete regions \( A \) and \( B \) of the conserved SNW domain and affected conserved/semi-conserved amino acid residues. In order to determine the cause of the temperature-sensitive phenotype, site-directed mutagenesis was used to re-create the amino acid substitutions at the \( A \) and \( B \) regions of \( PRP45 \). The results revealed that changing the Prp45p structure by introducing mutations in both specific regions, disrupts this protein's ability to function at elevated temperature. The need to alter two regions simultaneously while modifications in either region are not sufficient for the Ts' phenotype, could be explained by the cooperative nature of these regions in supporting the function of Prp45p. More specifically, both regions could be parts of the same catalytic site/binding interface for nucleic acids or proteins and mutations in both of them determine a conformational modification that abolishes the biological function of the protein at elevated temperature.

\( prp45 \) Ts' mutants display only a mild step 2 splicing defect \textit{in vivo} and \textit{in vitro} at non permissive temperature. This does not seem to explain the observed growth defect at 37°C, suggesting that the mutations in regions \( A \) and \( B \) affect the protein's function in other cellular processes. There are many reports of Prp45p homologues involvement in transcription pathways (Zhang \textit{et al.}, 2003; Zhou \textit{et al.}, 2000). However, the gene reporter assay employed here to analyse a possible effect on transcription of \( prp45 \) did not show that these mutants caused such a defect. Further investigations of these mutants included microarray analyses that detected accumulation of snoRNA-containing species in both mutants at the non-permissive temperature. Moreover, subsequent Northern blottings confirmed the results and showed for these specific snoRNA host gene transcripts that they accumulate as intron lariat-exon 2 intermediates, a phenotype specific for mutants defective in the second step of splicing.

Following the analyses described here, other investigations will aim to add additional clues to the role of Prp45p in splicing, transcription and other cellular processes.
7.2 Prp45p role in the second step of splicing

Although the splicing defect was not very strong in \textit{prp45} Ts' mutants, it provided a first clue of \textit{PRP45} involvement in the second step of splicing; results are confirmed by analyzing the splicing defect of the snoRNA-containing genes \textit{in vivo}. The defect \textit{in vivo} should be further examined using a more sensitive method, primer extension assay, with primers complementary to exon 2 and intron of the snoRNA-intron encoded genes.

Furthermore, the analyses should be extended to the whole yeast intron-containing transcripts by using the microarray assay with different primers called "branchmers" (Spingola \textit{et al.}, 1999). They prime across the 2'-5' junction of the lariat and the cDNAs resulting from the reverse transcription correspond only to the lariat intron containing species, i.e. lariat-intron exon 2 intermediate or lariat intron. These species would specifically hybridize on the microarray to the intron probes and, if it is the case, to the intron contained-snoRNA probes. In this way the microarray assay would distinguish between step I mutants and step II/ post-step II splicing mutants. Step I mutants accumulate pre-mRNA species in which signals are emitted by 5' splice site probes (V), step II mutants (and after) accumulate lariat intron–exon 2 and lariat intron species. The method has already been used in our lab for a Ts' mutant of Prp26p/ Dbrlp which is the lariat debranching enzyme (Chapman and Boeke, 1991) (D. Barrass, personal communication).

The effect of Prp45-113p/Prp45-57p inactivation on the second step of splicing could be further examined by determining whether c-Myc-tagged mutant proteins could co-immunoprecipitate, upon heat inactivation, the splicing intermediates. Previous analyses showed that Prp45p co-precipitated the pre-mRNA, the lariat intron-exon 2, and exon 1 intermediates as well as the excised intron (Albers \textit{et al.}, 2003). Therefore it would be interesting to investigate whether the mutants are unable to precipitate these species, indicating a defect.
7.3 Additional interactors of Prp45p

Subsequent two-hybrid screens performed at Hybrigenics with Prp46p as bait identified Prp45p as a main interactor, confirming the previous results of Albers et al. (2003), and narrowed down the minimum region of interaction, within Prp45p amino acid boundaries 55 and 98, quite distant from regions A (spanning between amino acids 120 – 130) and B (located within amino acids 190-211). Further in vitro analyses performed in this work showed that Prp46p co-precipitated with the two Prp45p Ts' mutants, indicating that the regions A and B are not involved in this interaction. Therefore it is quite likely that other interactors of Prp45p are still needed to be identified and this could be done using the two Prp45p Ts' mutants in genetic screens.

One possibility is to screen for suppressors by subjecting the mutant strains to chemical mutagenesis and look for colonies that lose the Ts' phenotype at elevated temperature but which confer another phenotype that is independent of the initial one (for example, cold-sensitivity). The identity of the suppressors can be established by transforming the mutant strains with a library and selecting for the loss of cold-sensitivity at 16°C.

Dominant suppressors constitute another option to identify putative interactors. Over-expressing a wild-type gene located on a high-copy number plasmid either in prp45-113 or prp45-57 strain and looking for the loss of temperature-sensitivity at 37°C, is an indication that the interaction with a wild-type protein has been restored or the need for this interaction has been bypassed.

Another genetic approach to identify Prp45p interactors is to isolate synthetic lethal mutants to prp45 Ts' mutations. This technique was used successfully in isolating Snt309p in a prp19 Ts' synthetic lethal screen (Chen et al., 1998) and lately mutants synthetically lethal with clfl Ts' mutant (Vincent et al., 2003).
Alignment of the amino acid sequences of SNW proteins revealed that SNWKN motif is not 100% conserved as was considered previously. Indeed the first asparagine residue of this motif seemed to be substituted for a methionine in the simple parasite *E. cuniculi* (Figure 1.). In an attempt to determine the functional significance of this motif, Martinkova et al., (2003) mutagenized the yeast SNW sequence to AAA and found that the modifications did not alter the protein function or, as present work demonstrated, mutations only in one domain of Prp45p were not incompatible with cell viability. Therefore, additional site-directed mutagenesis on yeast Prp45p could be used to make other modifications (substitutions with alanine residues) in motifs that are 100% conserved among the members of the SNW family (*prp45-113* and *prp45-57* contain mutations affecting absolutely conserved amino acids, K133E and L208S).

Solving the Prp45p crystal structure would provide further information about the positions where these mutations could be engineered. The crystal could be obtained with recombinant Prp45 protein produced in *E. coli* from an expression vector constructed in our lab (M. Albers, PhD Thesis). This crystallisation could give information about the secondary and tertiary structure and would also give clues about how different mutations might affect the protein conformation. For example it could clarify the functional significance of region *A* and region *B* within the general conformational structure of Prp45p.

To date this analysis has not been done for any member of the SNW family; therefore solving the crystal of Prp45p would open new possibilities for the investigation of this protein family.

The random mutagenesis screen identified one Prp45p Ts' mutant (P-3) that is an N-terminal truncated form of the protein retaining the first 47 amino acid residues. To investigate the biological importance of the N-terminal domain, a tagged version of
this truncated Prp45p could be constructed in BMA38a and analyses similar to the one performed for prp45-113/prp45-57 could be employed.

7.5 Prp45p role in transcription

prp45 mutants studied here did not confer a transcription defect to the cells, at least for the β-galactosidase transcript which was tested in section 6.3. To extend this analysis, the mutants could be used in a microarray assay having PAN Array slides provided by MWG which contain all the yeast transcripts, to look for a gene-specific effect.

Prp45p is definitely a very interesting protein with a structure different from its orthologs and having at least one function besides pre-mRNA splicing as found for its homologues. The presence of SH2 domain, the putative phosphorylation sites and interaction with Pkc1 found in two-hybrid screens make it worthy of further study as a potential target for regulation of splicing and/or transcription.
Chapter 8  Bibliography


Identification and characterization of Prp45p and Prp46p, essential pre-mRNA splicing factors

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ABSTRACT

Through exhaustive two-hybrid screens using a budding yeast genomic library, and starting with the splicing factor and DEAH-box RNA helicase Prp22p as bait, we identified yeast Prp45p and Prp46p. We show that as well as interacting in two-hybrid screens, Prp45p and Prp46p interact with each other in vitro. We demonstrate that Prp45p and Prp46p are spliceosome associated throughout the splicing process and both are essential for pre-mRNA splicing. Under nonsplicing conditions they also associate in coprecipitation assays with low levels of the U2, U5, and U6 snRNAs that may indicate their presence in endogenous activated spliceosomes or in a postsplicing snRNP complex.

Keywords: Protein interaction; snRNP; spliceosome; two-hybrid; yeast

INTRODUCTION

The excision of introns from precursor messenger RNAs (pre-mRNAs) occurs by two consecutive trans-esterification reactions in the spliceosome, a large and highly dynamic, ribonucleoprotein complex. In many in vitro studies, spliceosomes have been observed to assemble de novo on each substrate pre-mRNA in an ordered stepwise manner. In a series of ATP-dependent steps, five small RNAs, the U1, U2, U4, U5, and U6 snRNAs, in the form of small ribonucleoprotein particles (snRNPs), plus a variety of non-snRNP proteins associate with the pre-mRNA (for review, see Burge et al. 1999; Will and Lührmann 2001). Prior to activation of the spliceosome and during the splicing process, a number of conformational rearrangements take place, including critical changes to RNA–RNA interactions (for review, see Nilsen 1998). These rearrangements include dissociation of an extensively base-paired U4/U6 snRNA heterodimer, displacement of a U1 snRNA:5′ splice site interaction, and a consequent loosening of the associations of the U1 and U4 snRNPs with the spliceosome immediately prior to the initiation of the splicing process. Following the completion of the two splicing reactions, the spliceosome appears to dissociate, at least in vitro.

The splicing factors have been identified through genetic approaches in the budding yeast Saccharomycyes cerevisiae (for review, see Beggs 1995), fractionation of HeLa cell nuclear extracts and reconstitution of activities (Caceres and Krainer 1997), two-hybrid screens (e.g., Fromont-Racine et al. 1997) or by affinity purification of complexes under mild, nondenaturing conditions and identification of the protein components by mass spectrometry (Neubauer et al. 1997, 1998; Stevens and Abelson 1999). Using the latter approach, Stevens et al. (2002) isolated a large penta-snRNP complex that contains all five spliceosomal snRNAs and over 60 protein splicing factors. This complex supports pre-mRNA splicing when complemented with soluble factors, and it was proposed that the spliceosomal snRNPs may associate to form a penta-snRNP complex independently of the substrate pre-mRNA (for review, see Nilsen 2002).

In addition to aiding the identification of novel splicing factors, two-hybrid screens provide information about interactions between known splicing factors, and can also define regions of interaction between two binding partners (e.g., Fromont-Racine et al. 1997; Ben-Yehuda et al. 2000). Through exhaustive two-hybrid screens using a budding yeast genomic library and starting with the DEAH-box RNA helicase Prp22p as bait, we identified yeast Prp45p and Prp46p. We show that Prp45p and Prp46p interact with each other in vitro. We demonstrate that Prp45p and Prp46p are spliceosome associated throughout the splicing process and both are essential for pre-mRNA splicing. Un-
der nonsplicing conditions they associate in coprecipitation assays with a low level of U2, U5, and U6 snRNAs that may indicate their presence in endogenous activated spliceosomes or, more likely, in a postsplicing snRNP complex. These data are compatible with the recent detection of these proteins in the penta-snRNP complex (Stevens et al. 2002) and in another high molecular weight complex that contains a subset of spliceosomal proteins (Ohi et al. 2002).

RESULTS

Two-hybrid analysis of Prp45p

With the aim of identifying novel splicing factors and to shed light into the myriad of protein–protein interactions within the spliceosome, a number of two-hybrid screens of a yeast genomic library (FRYL; Fromont-Racine et al. 1997) were performed. Using the full-length Prp22 protein of S. cerevisiae as bait, 47 million potential interactions were tested, and a total of 20 clones were identified as positive for activation of both of the reporter genes used (Table 1). Ten isolates encoded five independent fragments of Prp45p (ORF YAL032c). All the Prp45p prey fragments share a small common region between amino acids 626 and 687 (Fig. 1A), which therefore corresponds to a minimum region of interaction although it is not clear that this is sufficient for the interaction.

A second candidate interactor, Syf3 protein, was isolated in the Prp22p two-hybrid screen a total of five times as three independent fusions. The C-terminal region, consisting of amino acids 628 to 687, corresponds to the minimum region of Prp22p interaction in Syf3p (Fig. 1B). Syf3p (also called Clf1p) was shown earlier to be involved in spliceosome assembly as well as in cell cycle progression from G2 to M phase (Chung et al. 1999; Russell et al. 2000).

Full-length Prp45p was used in turn as bait to screen the FRYL genomic library, and 34 million potential interactors were tested. By far the most statistically significant interactor was the protein Prp46 encoded by ORF YPL151c (Table 1); 19 plasmids encoding six independent protein fragments were isolated (Fig. 1C). The Prp45p interacting region appears to be within the amino acid boundaries 127 and 432 of Prp46p, as deduced from the overlapping region of the isolated fragments. This region encodes seven copies of a conserved WD repeat motif that is thought to mediate protein–protein interactions (Smith et al. 1999). None of the other prey isolated in this two-hybrid screen corresponds to a WD repeat protein. Prp46p is the yeast homolog of the recently identified human splicing factor PLRG1 and the Schizosaccharomyces pombe protein prp5, for both of which association with subspliceosomal complexes and their requirement for pre-mRNA splicing has been demonstrated (Potashkin et al. 1998; McDonald et al. 1999; Ajuh et al. 2000, 2001).

Interestingly, the Prp45p bait also found a total of six isolates of Syf3p. These represented two independent fragments of Syf3p that are situated close to the N terminus, sharing a common region between amino acids 35 and 156 (Fig. 1B). Notably, this region is different from the Prp22p interacting region, which is located at the extreme C terminus of Syf3p.

In addition, another known splicing factor with a cell cycle link, namely the Syf1 protein (Russell et al. 2000), was found to interact with the Prp45p bait. This interaction is statistically less significant, possibly indicating that this interaction is weaker or indirect (Table 1).

In conclusion, these results suggest an association of Prp45p with four known or potential splicing factors, although this two-hybrid assay may not distinguish between direct and indirect interactions.

Depletion of Prp45p causes a splicing defect in vivo

The two-hybrid associations of Prp45p with a set of splicing proteins led us to ask whether Prp45p is also required for pre-mRNA splicing. Because it was reported earlier (Harris et al. 1992) that Prp45 (FUN20) is a gene essential for cell viability, we constructed strain YMA45/2 with a galactose-inducible and protein A-tagged chromosomal Prp45 allele. Cultures of YMA45/2 and of the parental wild-type strain BMA38a were transferred to either permissive (galactose) or nonpermissive (glucose) conditions, and growth and pre-mRNA splicing were monitored. About 8 h after the transfer of the cultures to the nonpermissive conditions, the growth rate of YMA45/2 cells started to decline, and growth stopped at around 12 h (data not shown), unlike wild-type cells and YMA45/2 cells grown under permissive conditions, which continued to grow logarithmically. Northern analysis (Fig. 2A) showed that unspliced RP28 RNA accumu-
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<sup>a</sup>The open reading frames that were identified as prey in two-hybrid screens with full-length Prp22p, Prp45p, and Prp46p bait fusions. Indicated are systematic protein and ORF names as given in the Saccharomyces Genome Database.

<sup>b</sup>MRI: minimal region of interaction—amino acid boundaries of the region shared by all isolates of a given protein (* C termini of these inserts have not been determined).

<sup>c</sup>Isolates: total number of prey isolates.

<sup>d</sup>Category: Prey fusions were classified as defined by Fromont-Racine et al. (1997). A1 (statistically the most significant): multiple coding fusions that share a common region; A2: fusion starts close to the translation initiation codon; A3: contains a large coding insert; A4: single coding fusion not represented in A2 or A3 category, and possibly the least significant, although this may also indicate that only a precisely defined peptide can mediate an interaction.
The RP28 pre-mRNA intron, which was assayed by Northern analysis, has a branchpoint–3' splice site distance of 39 nt. To test whether Prp45p might be dispensable for splicing of introns with very short branchpoint–3' splice site distances, splicing of U3 pre-snoRNA was investigated, in which the distance between the branchpoint sequence and the 3' terminal CAG is only 7 nt. Figure 2B shows the results of primer extension analysis of U3 RNA and, as a control, of U1 snRNA. Already after 6 h growth under nonpermissive conditions, there was a strong accumulation of the two U3 precursor RNAs in strain YMA45/2, but not in the control cultures. Thus Prp45p may be required for the splicing of all spliced introns.

**Spliceosomal association of Prp45p**

To determine whether and at what particular stage of the splicing process Prp45p interacts with spliceosomes, the analysis was performed as described above. The RP28 pre-mRNA intron, which was assayed by Northern analysis, has a branchpoint–3' splice site distance of 39 nt. To test whether Prp45p might be dispensable for splicing of introns with very short branchpoint–3' splice site distances, splicing of U3 pre-snoRNA was investigated, in which the distance between the branchpoint sequence and the 3' terminal CAG is only 7 nt. Figure 2B shows the results of primer extension analysis of U3 RNA and, as a control, of U1 snRNA. Already after 6 h growth under nonpermissive conditions, there was a strong accumulation of the two U3 precursor RNAs in strain YMA45/2, but not in the control cultures. Thus Prp45p may be required for the splicing of all spliced introns.
The association of protein A-tagged Prp45p (protA:Prp45p) with pre-mRNA, intermediates, or products of splicing was tested by coprecipitation. Whole-cell extract produced from strain YMA45/2 grown under permissive conditions (i.e., producing protA:Prp45p) was incubated under splicing conditions with radiolabeled actin pre-mRNA. An aliquot (10%) of each sample was analyzed for splicing activity ("splicing"), while the remainder was incubated with IgG-agarose ("I") or, as controls, agarose beads ("B") or anti-Prp8p antibodies ("C"). Prp8p is a U5 snRNP protein that is associated with the spliceosomes throughout the splicing reactions and should therefore precipitate the pre-mRNA, intermediates, and products of the splicing reaction as shown previously by Teigelkamp et al. (1995b). To assess background precipitation by the protein A epitope, extract was prepared from strain BMA38a (pNOPPATAIL) in which the protein A epitope alone ("protA") was produced. To aid the determination of the particular stage of the splicing process at which Prp45p associates with the spliceosome, the precipitations were also performed with extracts to which a purified recombinant form of dominant negative (dn) Prp2p ("c+Prp2Tp") had been added, which stalls splicing prior to the first step (Plumptre et al. 1994).

Compared with the Prp8p control (Fig. 3, lane 6), protA:Prp45p efficiently coprecipitated ("colIP"; Fig. 3, lane 4) the pre-mRNA, the lariat intron-exon 2 (LI-E2), and exon 1 (E1) intermediates as well as the excised intron (LI; lane 4). As also observed for Prp8p, spliced mRNA was not enriched in the protA:Prp45p precipitate, although some coprecipitation of mRNA cannot be ruled out, as detection of the mRNA was poor. In both negative controls, using agarose beads without antibody (Fig. 3, lane 5) and providing the protein A epitope but no Prp45p (Fig. 3, lane 21), only very low levels of pre-mRNA were found in the precipitate, demonstrating specific precipitation by Prp45p. These findings showed that Prp45p is associated with the spliceosome throughout the splicing reactions and that it stays associated with the spliceosome until after the second catalytic step, when it is found associated with the excised intron.

When splicing was stalled due to prior addition of Prp2LATp to the extracts (Fig. 3, lanes 7–9), the Prp45 protein still precipitated almost equivalent quantities of pre-mRNA compared to untreated extracts (Fig. 3, lane 10 compared to 4). Therefore, Prp45p is already in the spliceosome before Prp2p acts to promote the first transesterification reaction. Small amounts of precipitated splicing intermediates and excised intron from these stalled extracts must be the result of a low residual splicing activity in the extracts due to the presence of wild-type Prp2p.

These data demonstrate that Prp45p associates with the splicing machinery throughout the splicing process, and upon dissociation of the spliced mRNA, Prp45p may segregate with the excised intron.

**FIGURE 3.** Coprecipitation of spliceosomes by Prp45p. Whole yeast cell extract (splicing extract) was prepared from cells of strain YMA45/2 grown in galactose-based medium, producing protA:Prp45p. As a control, extract was prepared from the parental wild-type strain carrying vector pNOPPATAIL, producing a double protein A epitope (protA). Splicing (50 µL total volume) was performed using 32P-labeled actin pre-mRNA. The reactions were stopped and 5 µL were removed as splicing controls (input). The remaining 45-µL samples were mixed with an equal volume of precipitation buffer containing either IgG-agarose (I), agarose beads without antibody (B) or protein A-Sepharose beads with prebound anti-Prp8p antibodies (C) and incubated at 4°C for 2 h. Beads were washed in buffer containing 150 mM NaCl, deproteinized, and the RNAs precipitated. The samples from the immunoprecipitations (colIP) were then reuspended in formamide loading buffer, resolved on a 6% (w/v) polyacrylamide gel, and labeled RNAs were visualized by autoradiography. In additional samples, recombiant dominant negative Prp2p was added to the extract prior to splicing (+ Prp2LATp) and the samples were treated as above. The positions of the RNA species are indicated. (lariat I-E2) Lariat intron-exon2; (LI) Lariat-intron; (E1) exon1.

**Coimmunoprecipitation of spliceosomal snRNAs with Prp45p**

To investigate whether Prp45p is snRNA associated, coimmunoprecipitation of the spliceosomal U1, U2, U4, U5, and U6 snRNAs with protA:Prp45p was tested. Splicing extracts of strain YMA45/2 producing protA:Prp45p and, as a negative control, strain BMA38a-pNOPPATAIL were either incubated with IgG-agarose (I), or, as controls, with agarose beads (B) or anti-Prp8p antibodies, prebound to protein A-Sepharose beads (C). After the immunoprecipitation with IgG agarose, the coprecipitated snRNAs were detected by Northern analysis.

As shown in Figure 4, lanes 5 and 8, under normal precipitation conditions, washing with 150 mM salt, Prp8p strongly coprecipitated U4, U5(L and S), and U6 snRNAs, as expected for this U5 snRNP protein that is also present in U4/U6/U5 tri-snRNPs. Significantly, under these conditions, protA:Prp45p pulled down approximately equal amounts of the U2, U5, and U6 snRNAs, but no U1 or U4 snRNAs (Fig. 4, lane 6). Low amounts of U1 and U4 snRNAs were only detected when the precipitates were washed at lower stringency (75 mM salt; Fig. 4, lane 9). This suggests that Prp45p associates with U2, U5, and U6 snRNAs.
Among 14 spore tetrads that were dissected, none produced gene and the YMA151KO1, allele of deletion experiment was performed. involvement of Prp46p in splicing seemed likely. homologs, PRLGI and prp5, respectively (Potashkin et al. link with splicing for the putative human and fission yeast with Syf3p in the two-hybrid screens and considering the The finding that Prp46p interacted with Prp45p as well as snRNAs under nonsplicing conditions (no ATP was added to the extracts), and has a lower level of salt-sensitive interaction with U1 and U4 snRNPs.

Analysis of the Prp46p knockout

The finding that Prp46p interacted with Prp45p as well as with Sy3p in the two-hybrid screens and considering the link with splicing for the putative human and fission yeast homologs, PRLGI and prp5, respectively (Potashkin et al. 1998; McDonald et al. 1999; Ajuh et al. 2000, 2001), an involvement of Prp46p in splicing seemed likely. To approach a functional analysis of Prp46p, a gene deletion experiment was performed. A diploid strain, YMA151KO1, was constructed in which the ORF of one allele of PRP46 was entirely replaced by the HIS3 marker gene and the PRP46/prp46Δ::HIS3 strain was sporulated. Among 14 spore tetrads that were dissected, none produced more than two viable spores, all of which were His⁰ (data not shown), demonstrating that PRP46 encodes an essential gene in S. cerevisiae.

To facilitate further functional characterization of the essential Prp46 protein, strain YMA151/2 was constructed in which the chromosomal PRP46 gene was transcribed under control of the regulatable MET3 promoter, which is repressed by methionine. Cultures of YMA151/2 and parental strain BMA64α were grown either in the absence (permissive conditions) or in the presence (nonpermissive conditions) of 7 mM methionine. At various time points, RNA was extracted and assayed by primer extension analysis for splicing of precursor U3 snoRNA (Fig. 5).

Already after 4 h growth of strain YMA45/2 under nonpermissive conditions, just as the growth rate began to decline compared to the permissive state (Fig. 5A), accumulation of the U3A and U3B precursor RNAs was detectable (Fig. 5B, lane 2). Thus, repressing PRP46 expression, thereby depleting the protein from the cells, leads to a splicing defect that causes the accumulation of unspliced precursor RNAs. Therefore, we conclude that Prp46p is required for pre-mRNA splicing in S. cerevisiae.

Prp45p and Prp46p interact in vitro

To test whether the two-hybrid interactions between Prp45p and Prp46p could be reproduced in vitro, a coupled in vitro transcription–translation system was used to produce 35S-labeled Prp45p or fragments thereof and these were incubated with yeast extract containing HA₃-tagged Prp46p. As shown in Figure 6, HA₃:Prp46p coprecipitated full-length Prp45p as well as an N-terminal fragment, R1, that contains amino acids 1 to 218, but not fragments R2, R4, or R46, all of which include the minimum region of interaction (amino acids 54–204) determined in the two-hybrid screen. To investigate the region of Prp46p required for this interaction and to determine whether these proteins interact directly, 35S-labeled fragments of Prp46p were produced in vitro and incubated with His₆-tagged Prp45p that had been produced in Escherichia coli and affinity purified. As shown in Figure 7, His₆:Prp45p coprecipitated full-length Prp46p as well as fragments "a" and "b" that lack the C-terminal 19 and 24 amino acids, respectively. Neither fragments "e" nor "d" was coprecipitated with His₆-Prp45p, although they contain all seven WD repeats, and "d" corresponds to the minimum region of interaction determined in the two-hybrid screens. Other fragments "e", "f", "g", and "h" that start at the N terminus, are truncated at the C end, and contain 6, 5, 3, or no WD repeats also failed to coprecipitate with Prp45p (data not shown).

DISCUSSION

Prp45-like proteins have been identified and characterized in other organisms, including SKIP/SNW1/NCoA-62 in hu-
Prp46p is a member of the ancient family of WD repeat proteins that have diverse functions in a variety of cellular processes (Smith et al. 1999). Prp46p shares greatest similarity with a subset of these proteins that are likely to be functional homologs. This includes the human protein PLRG1, which was demonstrated to be a component of human spliceosomes (Neubauer et al. 1998) and essential for splicing in HeLa nuclear extract (Ajouh et al. 2001), and the S. pombe prp5 protein. Mutation of the prp5 gene results in pre-mRNA splicing defects as well as a block to cell cycle progression at the G2/M phase (Potashkin et al. 1998; McDonal et al. 1999). The related Arabidopsis thaliana PRL1 protein regulates glucose and hormone responses (Nemeth et al. 1998). All these proteins contain a C-terminally located cluster of seven WD repeat units that are thought to mediate protein–protein interactions.

The interactions described here of Prp22p with Prp45p, of Prp45p with Prp46p, and of both Prp45p and Prp46p with Syf3p in two-hybrid screens also suggested possible roles for budding yeast Prp45p and Prp46p in the splicing process, and we demonstrate that metabolic depletion of either Prp45p or Prp46p results in a splicing defect (Figs. 2 and 5). Compatible with a role as a splicing factor, protA:Prp45p and HA-tagged Prp46p coprecipitated low levels of U2, U5, and U6 snRNAs (Fig. 4; data not shown);

Prp45p coprecipitates with Prp46p in vitro. (A) 35S-methionine-labeled full-length Prp45p or fragments of the regions indicated were produced in vitro. (B) The in vitro-produced polypeptides were incubated with ATP-depleted yeast splicing extract containing HA-tagged Prp46p and with anti-HA antibodies on ice for 1 h before adding protein A-Sepharose beads. The pellets and supernates (1.6%) were analyzed by SDS-PAGE and the 35S-labeled Prp45 polypeptides were visualized by autoradiography.

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Syf3p associate with low levels of U5 and U6 snRNAs, and complexes is unclear.

However, both proteins were rarely present in the same complex, and the functional significance of these different interactions that is compatible with the existence of a complex (Stevens et al. 2002). The results presented here extend this network of interactions to include Prp45p, Prp46p, and Prp22p. Indeed, these correspond to a subset of the Cwc complex of proteins (McDonald et al. 1999; Ohi et al. 2002) and some of these proteins have now been shown to interact with each other as the predominant binding partners in two-hybrid screens, and both found Syf3p as a highly significant interactor (A1 category; Fromont-Racine et al. 1997). We have shown that in addition to their interaction in two-hybrid screens, Prp45p and Prp46p interact in vitro, most likely through direct protein–protein contact. In addition, the use of truncated forms of these proteins produced in vitro confirmed the approximate region of the proteins required for the interaction. In each case, however, the interaction required more of the in vitro produced proteins towards the N termini compared to the minimum regions found in the two-hybrid screens. As the two-hybrid prey are fusion proteins, fused at their N-terminal ends, this might simply reflect a requirement for additional sequence at the N termini of these proteins to promote correct folding and/or stability.

A number of proteins including Cef1p, Isy1p, Syf1p, Syf2p, and Syf3p have been shown to interact with each other and with Prp45p and Prp46p in two-hybrid screens and coprecipitation assays (Dix et al. 1999; Ben-Yehuda et al. 2000; this work) and have been detected together with Prp45p and Prp46p in large protein complexes, including the Cef1p-associated “Cwc” complex (Ohi et al. 2002) and a penta-snRNP complex that may represent a form of recycled spliceosome (Stevens et al. 2002). In a high-throughput mass-spectrometric analysis (Gavin et al. 2002), Prp45p and Prp46p were detected in multiple complexes that contained overlapping subsets of these and other proteins; however, both proteins were rarely present in the same complex, and the functional significance of these different complexes is unclear.

It was previously reported that Isy1p, Syf1p, Syf2p, and Syf3p associate with low levels of U5 and U6 snRNAs, and under some conditions also with U2 snRNA (Dix et al. 1999; Russell et al. 2000), and it was proposed that this might indicate an association with a postsplicing complex (Dix et al. 1999). The salt-sensitive association of Prp45p with lower levels of U1 and U4 snRNAs supports this hypothesis, as following activation of the spliceosome, these two snRNAs are less strongly associated with this complex. Like Prp8p, Prp45p coprecipitated the excised intron lariat (Fig. 3). Thus, these data may indicate the presence of Prp45p and Prp46p in a postsplicing complex that contains the excised intron and the U2, U5, and U6 snRNPs, although the coexistence of all these factors in a single complex is not demonstrated here. Interestingly, the Cef1p-associated “Cwc” complex that includes Prp45p and Prp46p also contains U2, U5, and U6 snRNAs, which was reported to indicate spliceosome association (Ohi et al. 2002).

Significantly, Prp45p and Prp46p found each other as the predominant binding partners in two-hybrid screens, and both found Syf3p as a highly significant interactor (A1 category; Fromont-Racine et al. 1997). We have shown that in addition to their interaction in two-hybrid screens, Prp45p and Prp46p interact in vitro, most likely through direct protein–protein contact. In addition, the use of truncated forms of these proteins produced in vitro confirmed the approximate region of the proteins required for the interaction. In each case, however, the interaction required more of the in vitro produced proteins towards the N termini compared to the minimum regions found in the two-hybrid screens. As the two-hybrid prey are fusion proteins, fused at their N-terminal ends, this might simply reflect a requirement for additional sequence at the N termini of these proteins to promote correct folding and/or stability.

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ously, Prp22p was not present in the penta-snRNP complex, although it was detected by mass spectrometry as a potential Cwc component (Ohi et al. 2002). Thus the two-hybrid interactions that we detected between Prp22p as bait and Prp45p and Ssf3p may represent transient interactions of Prp22p with these proteins in the spliceosome.

Analysis of the splicing of two different introns in cells that had been metabolically depleted of Prp45p indicated that, unlike Prp22p, Prp45p may be required for splicing irrespective of the different branchpoint–3’ splice site distances of the introns. This result argues against a possible role for Prp45p in directly modulating Prp22p function in 3’ splice site selection and cleavage, as in that case, one would expect to see a similar selective requirement for Prp45p only for splicing of introns with short branchpoint–3’ splice site distances. However, recent experiments using an intron-specific microarray to obtain an overview of the splicing of all pre-mRNAs in S. cerevisiae indicated that analyzing the effects of a mutant splicing factor on a few selected pre-mRNAs can be misleading (Clark et al. 2002).

Prp18p, previously reported to be dispensable for splicing introns with branchpoint–3’ splice site distances of 17 nt or more in vitro, showed no such correlation when the whole genome was analyzed in vivo. It remains a possibility that Prp45p could act as a general second-step splicing factor in close proximity or even in contact with Prp22p at a stage late in the splicing process. Indeed it is conceivable that Prp45p might activate Prp22p in its reported function of releasing the spliced mRNA from the spliceosome (Company et al. 1991), leaving Prp45p with the residual intron-containing postsplcising complex.

Although the A1 category prey (those that are found as multiple fusions) in a two-hybrid screen are considered to be statistically more significant than others (Fromont-Racine et al. 1997), prey that occur as single fusions may also be functionally significant and may not arise as multiple fusions if the N terminus of the protein is essential for the interaction (A2 category), if most of the open reading frame is required (A3 category), or if the interaction only works with a specific fragment of the protein, no more and no less (A4 category). When multiple two-hybrid screens are performed, such single prey fusions become more statistically significant if they are found in several screens with functionally related proteins as bait (but not as nonspecific prey with unrelated bait proteins). This is the case with the budding yeast protein kinase Pkc1p, which was found as an interactor with Prp45p (A3) and Prp46p (A3), as well as with Ctf1p (A1) and Syf2p (A4), as bait proteins (Ben-Yehuda et al. 2000; Table 1). Pkc1p has serine/threonine protein kinase activity, is required for signal transduction function in response to cell stress, and, like several of the splicing factors in this interaction network, is required to promote progression through the cell cycle from G2 to mitosis (Levin et al. 1992). Interestingly, the A. thaliana homolog of Prp46p, PRL1, acts as a heterologous receptor in vitro specifically for the protein kinase C-β II isoform (Nemeth et al. 1998). The yeast Prp46p and Prp45p sequences contain 14 or 7 potential Pkc-phosphorylation sites, respectively, as determined in a ScanProsite search, and when Neubauer et al. (1998) identified SKIP protein in assembled human splicing complexes, the protein separated into three distinct spots after two-dimensional gel electrophoresis, suggesting that SKIP might be subject to phosphorylation or other posttranslational modifications. It will therefore be interesting to determine whether the yeast Pkc1p interactions with spliceosome components represents a potential mechanism for regulating splicing through protein phosphorylation, with Prp46p and Prp45p being potential targets, or whether Prp46p simply acts as a facilitator for protein–protein interactions.

MATERIALS AND METHODS

Yeast growth media

Yeast minimal medium (YMM): 0.67% (w/v) Yeast nitrogen base without amino acids, 2% (w/v) glucose was supplemented with all amino acids except as indicated for auxotrophic selection. YMMRs: 0.67% (w/v) Yeast nitrogen base without amino acids, 2% (w/v) galactose, 2% (w/v) raffinose, 2% (w/v) sucrose was used for induction of the GAL1 promoter. YPD: 1% (w/v) Yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) glucose, 0.003% (w/v) adenine sulfate.

Strains

Yeast strains used in this work (diploid markers are homozygous unless indicated otherwise): BMA38 (MATa/α, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100), BMA38a (MATa, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100), BMA64α (MATα, his3-11,15, leu2-3,112, ura3-1, trp1Δ1, ade2-1) and the Clontech strains CG1945, L40, and Y187 for two-hybrid screens. Strain YMA45/1 (MATa/α, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, PRP45/HIS3-PGAL1-ProtA:PRP45) was made from BMA38 by integration of a linear DNA cassette encoding the HIS3 gene, GAL1 promotor, and two IgG binding sites of the Staphylococcus aureus protein A directly upstream of the PRP45 open reading frame. The cassette was generated by PCR from plasmid pTL27 (Lafontaine and Tollervey 1996) using oligonucleotides PFUN-1 and PFUN-2. Correct integration of the cassette was checked by PCR and inducible production of protein A-tagged Prp45p was monitored by Western blotting. Sporulation of YMA45/1 and subsequent selection for histidine prototrophic spores produced YMA45/2 (MATa, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, HIS3-PGAL1-ProtA:PRP45). BMA38a carrying pNOPPATAIL (gift from E. Hurt, Biochemie-Zentrum Heidelberg (BZH), Heidelberg, Germany), which encodes the double protein A epitope, was used as a control.

Strain YMA151KO1 (MATa/α, his3Δ200, leu2-3,112, ura3-1, trp1Δ1, ade2-1, can1-100, PRP46/PRP46Δ:His3) was constructed from BMA38 by complete replacement of the PRP46 open reading frame with a HIS3 cassette generated using oligonucleotides PLKO-1 and PLKO-2 as described in Baudin et al. (1993).
Strain YMA151/2 (MATa, his3-11,15, leu2-3,112, ura3-1, trplA1, ade2-1, TRP1-P_MT3-3A-HA2: PRP46) was generated from BMA46α by targeted integration of a linear cassette containing the TRP1 gene, the MET3-promoter and a double HA-epitope directly upstream of the PRP46 coding sequence. The cassette was produced by PCR from plasmid pUC19-HA55 (van Nues and Beggs 2001) with oligonucleotides 151MetA and 151MetB.

Oligonucleotides

PFun-1: 5'-TTACCTTAAAGCTATATTGTAATTCTTCAGAAT TTGATTTCCTGCGCCCTCTAGT-3'
PFun-2: 5'-CTTGAAGATTTGGTTGGAGGTGATCTGTTGTTAC TAAACTATCCGCTACTCTTCGCGG-3'
Func1: 5'-TAGATTTTCATGTTAAGGAGGACAGACGACG-3'
Func2: 5'-TAGGTCGACCTAGGCGCCATAGGGGTATTC-3'
PLK0-1: 5'-GAGGATGAGAAGCAGATGTAACTATGGAAGATT GGTGACCTTGGCGCTCTCTTAG-3'
PLK0-2: 5'-CACGCTTATACGGGTAGTACCTTTTCCTATCC TCCATGTCGTTAAGGACG-3'
151MetA: 5'-CCACACAAAATCAGAGTGACCTTAACACATTT GCCGTTAATTCCTTAAATATACAGCTC-3'
151MetB: 5'-TGTTGATGATCCTCTAAATTTTGAGCTTGTGAT CATTTGCGTCCATACGAGGCGGCTACTGGA-3'
151-PR2: 5'-GTATAATGACGGAAGTCC-3'
YPI151A: 5'-AAGGATGATGAGTTGGAATGTAGCAGCAGG-3'
YPI151B: 5'-AAGGATGATGTGAGCTCTCACTTACCTCCAC-3'
Met3: 5'-TTTTACCTTTTGATGTACTC-3'
PGK: 5'-ACCGCTTGTACTACCCCAATGGAGAAGGCAACAC-3'
RP28A: 5'-TCGACTTATGGCTCATTCC-3'
RP28B: 5'-TGAAAACCTTATACCTTTAC-3'
ant-U1: 5'-CACCAGCTTGTACGGCTG-3'
ant-U2: 5'-CTACACTTGATCTCAACGCAACAG-3'
ant-U4: 5'-CCGCTGTAAAGGAT-3'
ant-U5: 5'-AATATGGCAAAGCC-3'
ant-U6: 5'-TC(A/T)TCTCTGTATTTG-3'

Plasmids

To make the Gal4:PRP22p two-hybrid bait construct, plasmid pBSKS22 (gift from B. Schwer, Cornell University, NY) was digested with KpnI and blunt-ended by T4 polymerase treatment. After NdeI cleavage, the PRP22-containing fragment was ligated into NdeI/Smal-digested pAS2ΔΔ (Fromont-Racine et al. 1997). To construct the LexA:PRP45p two-hybrid bait plasmid, the PRP45 ORF was PCR-amplified from yeast genomic DNA using oligonucleotides Func1 and Func2. The PCR product was cleaved with EcoRI, SalI and ligated in frame into EcoRI/SalI-digested pBTM116 (Vojtek et al. 1993). The LexA:PRP46p two-hybrid bait plasmid was constructed by PCR amplification of the YPL151c ORF using oligonucleotides YPL151A and YPL151B as primers and cloning into BamHI-cleaved pBTM116, in frame with the preceding LexA sequence.

All constructs were verified by DNA sequencing, and protein expression was tested by Western analysis using anti-Gal4 DNA binding domain antibodies (Santa Cruz Biotechnology) and/or by complementation of a yeast strain bearing a mutation in the corresponding genomic sequence. To make pETMA45 (for His2:Prp45p production in E. coli), the PRP45 ORF was isolated from pMA45 as an EcoRI-SalI fragment, pET19b was digested with NdeI, and both were treated with Klenow and ligated together.

Two-hybrid screens

Two-hybrid screens were performed as described in Fromont-Racine et al. (1997). In the Prp22p screen, positives were selected for growth on YMM-LWH agar and tested for β-galactosidase activity in an X-gal agar overlay assay (Fromont-Racine et al. 1997). In addition, relative strength of reporter gene activation was determined in a β-galactosidase filterlift assay (Transy and Legrain 1995). In the Prp45p two-hybrid screen, positives were selected first on YMM-LWH agar plates, then suspended in microtiter-plates and replicated onto YMM-LWH agar containing 20 mM 3-aminotriazole (3AT; which increases the stringency of the His+ selection). One hundred fifteen clones that grew on 20 mM 3AT (and were analyzed further. The Prp46p screen was as for Prp45p but using 50 mM 3AT, and 34 clones were obtained.

Primer extension assay

Yeast total RNA was prepared using the method of Schmitt et al. (1990). Oligonucleotide primers were labeled with T4 polynucleotide kinase, and primer extension was performed and the products analyzed on a 6% (w/v) polyacrylamide gel as described (Beltrame and Tollnerve 1992).

Splicing extract preparation and in vitro splicing reactions

Preparation of yeast whole-cell extracts and in vitro splicing reactions were performed as described (Lin et al. 1985). Plasmid p283, which contains an AirI fragment of the yeast actin gene, was transcribed in vitro with T7 RNA polymerase and [α-32P]UTP to produce uniformly labeled splicing substrate (O'Keefe et al. 1996). Splicing reactions (5 μL for splicing activity or 50 μL for immunoprecipitation) were incubated at 23°C for 20 min, and the reaction products were fractionated on a 6% (w/v) polyacrylamide gel and visualized by autoradiography.

Western blot analysis

Proteins were fractionated by SDS-PAGE and detected using peroxidase-anti-peroxidase (PAP; Sigma) and ECL (Amersham) according to the manufacturer's instructions.
Immunoprecipitations

Immunoprecipitations of snRNPs and spliceosomes were performed as described (Teigelkamp et al. 1995a), using IgG-agarose (Sigma) or anti-Prp8 polyclonal antibodies (anti-8,6), and washes containing 150 mM NaCl (or, when indicated, 75 mM NaCl).

Northern analysis of snRNAs

Immunoprecipitates obtained using IgG-agarose (Sigma) or anti-Prp8 polyclonal antibodies (anti-8,6; Teigelkamp et al. 1995a) were washed, deproteinized by SDS/proteinase K treatment followed by phenol/chloroform/isoamylalcohol extraction, and the RNA was fractionated on a 6% polyacrylamide gel. The snRNAs were detected by Northern analysis using end-labeled oligonucleotides complementary to the U1, U2, U4, U5, or U6 snRNAs (Cooper et al. 1995).

Protein production in bacteria

Proteins were produced in E. coli BL21(DE3)pLysS cells grown at 23–30°C to an O.D.600 of 0.3–0.8 (optimal conditions determined for each protein). IPTG was added to 0.5–1 mM and cultures were incubated for 1-3 h to induce expression. Cell pellets were resuspended in chilled lysis buffer A (50 mM Tris-Cl at pH 7.5, 250 mM NaCl, 10% [w/v] sucrose) containing Sigma P8749 protease inhibitor cocktail, lysozyme was added to 0.2 mg/mL, and the extract was stirred on ice for 45 min. After addition of Triton-X-100 to 0.1% [v/v] and stirring on ice for 5 min, the lysate was centrifuged at 17,000 rpm at 4°C for 45 min and the supernate was stored at -70°C until metal affinity purification of the His-tagged proteins with Ni-NTA agarose (Qiagen).

In vitro synthesis of 35S-labeled proteins

PCR-generated templates were used in an in vitro transcription/translation system (Promega TNT T7 Quick for PCR) according to the manufacturer's instructions, using 0.37 MBq 35S-methionine (Amersham) for labeling.

Commmunoprecipitation of proteins

TNT reaction (6 μL) was mixed with 40 μL His-tagged PrP45 protein or BC100 (controls; 20 mM Tris-HCl at pH 7.9, 100 mM KCl, 20% [v/v] glycerol) and incubated at room temperature for 10 min followed by 1 h on ice. Antipentahtidinidine antibodies (Qia- gen) bound to protein A-Sepharose beads were added, or beads alone as a control, and samples incubated at 4°C for 2 h with mixing. Supernates were removed and beads were washed three times with IP300 (6 mM HEPES, 300 mM NaCl, 2.5 mM MgCl2, 0.05% [v/v] Nonidet P40) and suspended in 25 μL 2x SDS buffer (125 mM Tris at pH 6.8, 4% SDS, 200 mM DTT, 40% glycerol, 0.02% bromophenol blue). Samples were heated to 70°C and analyzed by SDS-PAGE (Nu-PAGE 4–12% Bis-Tris in MOPS buffer). Gels were dried before autoradiography.

For immunoprecipitation experiments using yeast extract, 25 μL of HA3-tagged PRP46 splicing extract was depleted of ATP by adding 2 mM glucose and incubating at 24°C for 20 min. 35S-labeled PRP45 polypeptides (5 μL TNT reaction) and 4 μL of anti-HA antibodies (12CA5, Roche) were added and the NaCl adjusted to 150 mM with IP300. The mixture was incubated on ice for 1 h before adding protein A-Sepharose beads. The samples were rotated at 4°C for 2 h, then washed three times with ice-cold IP150 (as IP300 but 150 mM NaCl). The pellets and supernates (1.6%) were suspended in 1× SDS-loading buffer and analyzed by SDS-PAGE as above.

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