A GENETIC AND BIOCHEMICAL ANALYSIS OF pre-mRNA SPLICING IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Pre-mRNA splicing requires interaction of *cis*- acting intron sequences with *trans* -acting factors: proteins and small nuclear ribonucleoproteins (snRNPs). The assembly of these factors into a large complex, the spliceosome, is essential for the subsequent two step splicing reaction. First, the 5' splice site is cleaved and free exon1 and a lariat intermediate (intron- exon2) form. In the second reaction the 3' splice site is cleaved the exons ligated and lariat intron released. A combination of genetic and biochemical techniques have been used here to study pre-mRNA splicing in yeast.

Yeast introns have three highly conserved elements. We made point mutations within these elements and found that most of them affect splicing efficiency *in vivo* and *in vitro*, usually by inhibiting spliceosome assembly.

To study *trans* -acting splicing factors we generated and screened a bank of temperature-sensitive (*ts*) mutants. Eleven new complementation groups (*prp17* to *prp27*) were isolated. The four phenotypic classes obtained affect different steps in splicing and accumulate either : 1) pre-mRNA, 2) lariat intermediate, 3) excised intron or 4) both pre-mRNA and intron. The latter three classes represent novel phenotypes. The excised intron observed in one mutant: *prp26* is stabilized due to protection in a snRNP containing particle. Extracts from another mutant: *prp18* are heat labile and accumulate lariat intermediate and exon1. This is especially interesting as it allows analysis of the second splicing reaction. *In vitro* complementation of inactivated *prp18* extracts does not require intact snRNPs. These studies have also shown the mutation to be in a previously unknown splicing protein. A specific requirement for ATP is also observed for the second step of splicing. The *PRP18* gene has been cloned and its polyadenylated transcript identified.

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INTRODUCTION

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The removal of introns, from the primary transcript occurs by RNA splicing. Three major types of RNAs (tRNA, rRNA and mRNA) are known to contain introns. There are two general questions concerning any splicing mechanism: first, how is the precise recognition and alignment of the splice junctions achieved in introns whose lengths vary from a few hundred nucleotides to several thousands of nucleotides, and second, what are the biochemical mechanisms of the cleavage and ligation reactions.

The specificity of the splice junction selection and the juxtaposition of the exons is achieved in Group I introns (e.g.,Tetrahymena rRNA, some yeast mitochondrial introns, choloroplast tRNA introns) and Group II introns (e.g. fungal mitochondrial introns) by the conserved secondary and tertiary structure of the intron . In group I splicing, the intron is excised in a RNA catalysed reaction. The catalytic RNA is the intron itself and splicing occurs in the presence of a guanosine cofactor. Splicing of Group II introns have also been shown to take place by self excision, and the excised intron is released as a branched lariat RNA, a structure also produced in nuclear pre-mRNA splicing (reviewed by Cech and Bass 1986).

The splice site selection in nuclear pre-mRNA is dictated only by short stretches of sequences at or near the splice junctions (Shapiro and Senapathy 1987). Splicing involves interaction of these cis-acting sequences with many cellular factors including proteins and small nuclear ribonucleoproteins (snRNPs) (reviewed in Sharp 1987 ; Maniatis and Reed 1987). The development of *in vitro* splicing systems from mammalian cell lines and from the yeast, *Saccharomyces cerevisiae* , has revealed a two step splicing reaction scheme for splicing (Fig.1a). First, cleavage at the 5' splice junction results in the formation of a lariat intermediate of intron-exon2 in which the first base of the intron is covalently linked by a 2'-5' phosphodiester bond to an internal adenosine near the 3' splice site. In the second step, the 3' splice junction is cleaved and the exons are ligated to give the mRNA and the lariat intron. Mechanistically, the reaction scheme is very similar to that of the self-splicing Group II introns; however, the nuclear pre-mRNA splicing machinery is more complex. In

splicing reactions studied *in vitro* the pre-mRNA and the splicing intermediates are associated with a large complex, termed the spliceosome (Brody and Abelson 1985 ; Grabowski *et al.*, 1985 ; Frendewey and Keller 1985). The spliceosome resembles the complexity of ribosomal subunits in that it consists of several RNAs and proteins. It is also clear now that at least five snRNPs (U1, U2, U4, U5 and U6), are required for splicing in both yeast and mammalian systems. The participation of at least one heterogeneous nuclear ribonuclear protein (hnRNP) is also indicated in pre-mRNA splicing.

Enumeration, identification and determination of the function of spliceosomal components are essential for the complete understanding of the splicing machinery and its operation. Biochemical fractionation of extracts derived from several organisms has provided a useful approach in such an analysis. The extensive genetic repertoire of the yeast *Saccharomyces cerevisiae* allows the isolation and the characterization of mutants in the splicing pathway. The analysis of such mutants and the isolation of the wild type gene product of these loci is a powerful ally of the biochemical studies. A combined genetic and biochemical approach has therefore been taken by several groups working with yeast, in their attempt to identify the factors involved in the assembly and function of the spliceosome.

ROLE OF CIS ACTING SEQUENCES

The sequences in the pre-mRNA recognized for the formation of the spliceosome are found near the boundaries of the intron. In higher eukaryotic pre-mRNA the introns are bounded at the 5' splice site by a consensus sequence of about eight nucleotides and at the 3' splice site by a poly pyrimidine (Py) stretch followed by an AG. The intron sequences utilized for the formation of the branch are variable, but the branch acceptor nucleotide is generally an A (reviewed in Padgett *et al.*, 1986 ; Green 1986). Considerable variation of these conserved elements is observed in metazoan introns. Correct splice site selection in meta-

zoan introns therefore requires discrimination between bonafide splice sites and the many sequences that resemble the splice sites.

The situation is different in yeast, where analyses of intron sequences have revealed three highly conserved elements required for splicing. These are the 5' consensus GUAPyGU, the 3' junction sequence PyAG, and the internal UACUAAC near the 3' splice site. The acceptor nucleotide for branch formation in lariats is the penultimate nucleotide of the highly conserved UACUAAC sequence (Domdey *et al.*, 1984 ; Rodriguez *et al.*, 1984). These elements delineate the intron borders in most yeast genes. Analysis of mutations in these sequences have demonstrated the importance, and in some cases the necessity, of these conserved residues for pre-mRNA splicing. Most mutations in these conserved intron sequences affect the reactions of splicing, lariat intermediate formation and exon ligation, to varying degrees. In contrast, mutations in the splice sites of higher eukaryotes usually result in the activation of cryptic splice sites.

At the 5' splice site, mutations in the 5' G or U allow the first step of splicing to proceed, thereby allowing the lariat intermediate formation, but not the second reaction of splicing (Newman *et al.*, 1985 ; Fouser and Friesen 1986 ; Vijayraghavan *et al.*, 1986). Activation of cryptic 5' splice sites, rarely seen in yeast splicing systems, occurs upon mutation of the fifth nucleotide in GUAPyGU to a A or C (Parker *et al.*, 1985 ; Fouser and Friesen 1986). Hence, the GU dinucleotide does not seem to be essential for the precise cleavage, but the fifth nucleotide plays a role in the precision of the 5' splice site selection. These 5' splice site mutations which cause cryptic splice site utilization, result in the formation of dead end lariat structures.

Mutations in most nucleotides of the branch acceptor sequence inhibit the first step of the splicing, leading to accumulation of pre-mRNA. Mutations at the highly conserved branch acceptor nucleotide, A, do not alter the site of branch point formation. When the branch acceptor nucleotides is a C, inefficient splicing is observed (Fouser and Friesen 1986 ; Vijayraghavan *et al.*, 1986). However, G functions as a better branch acceptor than

C, but completely inhibits the second reaction of splicing (Fouser and Friesen 1986). These results suggest that the structures of the branch site play a significant role in the second reaction of splicing.

Unlike metazoan introns, the 3' splice site in yeast is determined only by the PyAG sequence and no equivalent of the polypyrimidine stretch is found. The 3' splice site mutations do not affect the first step of splicing, suggesting that they play little role in the early events of splicing. In fact, sequences downstream of the UACUAAC sequence and the 3' splice site do not seem to be required for the first step of splicing (Cellini *et al.*, 1986 ; Rymond and Rosbash 1985) in contrast to the critical role played by the polypyrimidine stretch in metazoan introns (Wieringa *et al.*, 1984 ; Frendewey and Keller 1985). Point mutation in the conserved AG sequence of yeast intron affects only the second step of the splicing reactions (Vijayraghavan *et al.*, 1986). These results indicate that, in general, the sequence elements in yeast and metazoan introns function in slightly different ways. The requirements in yeast for lariat intermediate formation are the presence of a proper 5' splice junction and a branch acceptor site. Sequences downstream of the UACUAAC are not required for lariat formation, but a minimal RNA length downstream of the UACUAAC sequence is required for the first step of splicing (Rymond *et al.*, 1987). The second step of splicing requires a PyAG 7 to 55 nucleotides downstream of a suitable branch structure. Intron sequences besides the conserved elements play a role in affecting the efficiency of splicing *in vivo* (Pikielny and Rosbash 1985).

YEAST SPLICEOSOMAL snRNA

Studies on the snRNAs (small nuclear ribonucleic acids) from metazoans have focussed on the abundant ones (namely U1 through U6 RNAs), which range in size between 90 and 216 nucleotides (nts). These snRNAs are usually found in the form of ribonucleoprotein complexes (snRNPs). Each of these U class snRNPs generally consists of a single RNA complexed with about six to ten proteins. The U1 to U6 snRNPs all belong to the Sm

class, which contain a highly conserved set of proteins recognised by a set of human autoimmune antibodies. The Sm antigen is believed to bind to a consensus $AU_{4-6}G$ motif in a single stranded region of the RNA. The U4 and U6 snRNAs are complexed in the same snRNP. The association between the U4 and U6 RNAs is due to base pairing interaction between the two RNAs. Oligonucleotide-directed RNase H cleavage of U1, U2 , U4, or U6 inhibits splicing reactions *in vitro* , implying a role for these snRNAs in splicing (reviewed in Maniatis and Reed 1987). Based on ribonuclease protection experiments, U1 binds the 5' splice site and U2 to the branch formation site. These experiments also suggest that a snRNP, possibly U5, recognises 3' splice site. No specific binding site for the U4/U6 snRNP has been identified yet. The hypothesis of pre-mRNA-U1 interaction through base pairing was first suggested on the basis of sequence complementarity between the 5' terminal nucleotides of U1 and the 5' splice site (Lerner *et al.*, 1980 ; Rogers and Wall 1980). This model has genetic support as splicing of 5' splice site defective pre-mRNA can be restored by compensatory base mutations in the U1 RNA (Zhuang and Weiner 1986).

A search for snRNAs in yeast by the criterion of the presence of the characteristic trimethyl cap structures has identified a set of at least 24 snRNAs that show a great variation in size from 100 to 1200 nt (Riedel *et al.*, 1986). The striking differences from the well characterised metazoan U class snRNAs are their low abundance of only 10-1000 copies per cell and the fact that they are encoded by single copy genes. Several of the initially analysed snRNAs of yeast were non-essential and these results were unexpected and made it difficult to establish a link between pre-mRNA splicing and snRNA in yeast .

Some of snRNA genes (called SNR) have now been found to be essential for growth; they include SNR7, SNR14, SNR19, SNR20, and SNR6 (reviewed in Guthrie and Patterson 1988). By a variety of other criteria this very same subset of essential snRNA was thought to be spliceosomal snRNAs. The structure of the first yeast U analog (U2 snRNA), proved to be a surprise due to the fact that it was six times larger than its

mammalian counterpart -- 1175 versus 189 nts (Ares 1986). Extensive homology at the 5' terminus of the yeast U2 RNA was found to the mammalian U2 snRNA. In addition to the homology to the mammalian U2, the yeast U2 also has internal domains with homology to mammalian U4, U5, and U6 snRNA. The theory that these domains could make this long snRNA from yeast a poly-snRNA equivalent, no longer holds for two reasons. Deletions removing large portions of the U2 snRNA are viable, and indispensable equivalents to the U4, U5, U6 snRNA have been found in yeast. Strains containing deletions that remove all but the domain with U2 homology and a stretch of ~100nts at the 3' end of the yeast U2 snRNA gene are viable (E. Shuster and C. Guthrie, pers. comm.). This leaves the functional domain as the region with homology to U2. Cloning and sequencing of other yeast spliceosomal snRNA indicates that yeast snRNAs in general tend to be larger than their mammalian equivalents.

The question of whether recognition by the U2 snRNP of the branch point sequence is due to base pairing has been tested genetically in yeast (Parker *et al.*, 1987). Supressor mutations in the SNR20 gene (which codes for the yeast U2 snRNA) were made to two point mutations in the UACUAAC actin intron sequence. The presence of the U2 supressor plasmid in a strain with mutant UACUAAC sequence integrated in the chromosome shows complete supression of both the growth and the splicing pattern. The supression was allele specific. The base pairing interaction between nucleotide near the 5' end of U2 and the branch sequence of yeast introns requires bulging out of one of the two adenosine nucleotides at the 3' end of UACUAAC. It has been suggested that the branch nucleotide be bulged out (Fig 2). This achieves the maximum number of base pairing interactions between the two RNAs and this also provides a similarity to the autocatalytic GroupII introns, where the lariat formation occurs at a bulged out A of a conserved element.

Secondary structure analysis has been able to define an essential yeast homolog to the mammalian U5 snRNA (Patterson and Guthrie 1987). This homology is found in a 70

nt domain with limited sequence homology but striking structural homology to the higher eukaryotic U5 snRNAs. Construction of a SNR7 gene under the control of an inducible promoter has allowed analysis of the biochemical phenotype when U5 snRNA is depleted in the cell. Accumulation of unspliced pre-mRNAs is seen after five generations of shift to uninduced conditions, demonstrating that U5 is essential for splicing.

Yeast U4, which is also Sm precipitable, is 160nt long and has several stretches of similarity to mammalian U4 and a similar secondary stucture (Siliciano *et al.*, 1987). The most striking conservation is in the 5' half of the molecule. Native gel electrophoresis of the snRNA from yeast has revealed an interaction of U4 with a second RNA designated U6. This RNA has primary sequence homology to U6 from humans and other organisms and is the most highly conserved spliceosomal snRNA. The U4 and U6 of yeast are associated in a complex with a melting temperature of 53C, suggesting extensive base pairing between the two RNAs (Brow and Guthrie 1988). This stable association indicates that an active mechanism must exist for the destabilization of the U4/U6 that is observed in *in vitro* splicing reactions (discussed in a later section).

The yeast U1 analogue although much longer than U1, has several regions of homology to U1. The 10 nucleotides at the 5' end are completely conserved when compared with the 5' end of U1 snRNA (Kretzner *et al.*, 1987 ; Siliciano *et al.*, 1987). This strict sequence conservation between the U1snRNAs implies the generation of a one base mismatch between yeast U1 and the almost invariant GUAPyGU at the 5'splice site of yeast introns (Fig 2). Base pairing contributions between U1 and the 5' splice site had already been shown in mammalian systems (Aebi *et al.*, 1987 ; Zhuang and Weiner 1987). Similar models have been tested in yeast, and these results are particularly interesting in the light of the unexpected lack of complete complementarity between the 5' splice site and the U1 snRNA. Recent evidence has demonstrated base pairing in at least two positions. In contrast to mammalian systems, precision at the 5'splice site is not determined solely by base pairing interactions with the U1 snRNP (P. Siliciano and C. Guthrie, per. comm.).

THE SPLICEOSOME AND ITS ASSEMBLY

The bimolecular structure (lariat intermediate and exon1) of the intermediates of pre-mRNA splicing strongly suggest the existence of a multicomponent structure on which pre-mRNA splicing takes place. Such a complex would hold together the two RNAs for the second step of the process. Splicing complexes were first detected upon sedimentation of *in vitro* reactions in glycerol gradients, whereupon a co-sedimentation of the splicing intermediates in a 40-60S particle was observed. This particle was named the spliceosome. The formation of the spliceosome was found to be necessary for the splicing reaction, and its assembly requires the presence of appropriate 5' splice site and 3' splice site sequences in mammalian systems; and in yeast, of the 5' and the UACUAAC sequences. A requirement for ATP was also observed in both systems *in vitro* (Brody and Abelson 1985 ; Grabowski *et al.*, 1985 ; Frendewey and Keller 1985 ; Vijayraghavan *et al.*, 1986). Depletion or inactivation of hnRNP proteins (Choi *et al.*, 1986) or snRNPs from the HeLa extracts (Frendewey and Keller 1985 ; Grabowski *et al.*, 1985) abolishes the formation of this complex.

A higher resolution of separation of the assembly intermediates and the spliceosome than that obtained on glycerol gradients was achieved by running splicing complexes on non-denaturing polyacrylamide gels. Heparin or total RNA was added to remove non-specific aggregation to the splicing complexes. The studies with yeast and mammalian *in vitro* reactions on these gel systems demonstrated the presence of three splicing-dependent, kinetically ordered complexes (Konarska and Sharp 1986 ; Pikielny *et al.*, 1986). An early complex of U2 and pre-mRNA whose formation is ATP dependent is followed by the appearence of a complex with U2, U4, U5, and U6 containing the pre-mRNA. The splicing intermediates were found in the third complex, which surprisingly did not have any U4, indicating a destabilization and exit of the U4 from the U4/U6 snRNP complex and the spliceosome. It was thus unclear if the U4 snRNA leaves the spliceosome before, after or during the first step of the splicing reaction. A fourth splicing complex containing U2, U4,

U6, U5 (complex A2-1 in Fig 1b and Fig 3) and only the pre-mRNA was subsequently found (Cheng and Abelson 1987 ; Lamond *et al.*, 1988). The detection of this complex demonstrated that before the formation of an active spliceosome, U4 snRNP left the splicing complexes. The complex containing the splicing intermediates (active spliceosome) was kinetically the last to form. Similar to the results from mammalian systems the U1 snRNP was not detected in any of these non denaturing gel complexes. Of the products of the reactions, the lariat intron is associated with a complex of U5, U6 and U2 snRNPs in nondenaturing gels of splicing reactions with Hela extracts (Konarska and Sharp 1987). The other product, the mRNA, is not associated with any snRNP but is probably associated with some hnRNPs and is transported to the cytoplasm.

The formation of the kinetically early assembly intermediate of pre-mRNA and U2 snRNP requires the addition of ATP. In HeLa cell extracts the U2 binding requires both the polypyrimidine and the 3'AG (Ruskin and Green 1985) . Analysis of double mutants with sequence alterations in both the 5' and the 3' splice sites of mammalian introns reveals that an interaction between the 5' and the 3' splice site promotes formation of the U2 containing complex (Lamond *et al.*, 1987). Mutations in the yeast 5' splice site or the UACUAAC that cause accumulation of pre-mRNA in splicing reactions, prevent even the binding of U2 snRNP (Cheng and Abelson 1987). The length of the pre-mRNA *per se* influences later stages of the spliceosome assembly (Rymond *et al.*, 1987). Substrates with short RNA stretches after the branch consensus sequence fail to undergo even the first reaction of splicing. Accumulation of early complexes containing only the pre-mRNA is seen, indicating that the failure to form lariats is due to a specific assembly defect. Mutations in the yeast 3' splice site consensus sequences that allow lariat intermediate accumulation in splicing reactions accumulate the late spliceosome with U2, U5, and U6 (Cheng and Abelson 1987). A more recent study on the effect of chemical modification and interference pattern of the pre-mRNA on the assembly and maturation of spliceosomes and on the splicing reaction has been conducted (Rymond and Rosbash 1988). This study

indicates that regions of the pre-mRNA sequence that are essential for splicing include, but are not limited to, the conserved intron sequences.

Analysis of the spliceosomal constituents using affinity chromatography with polyA$^+$ pre-mRNA in splicing reactions or with biotinylated pre-mRNA has been done (Pikielny and Rosbash 1986 ; Grabowski and Sharp 1986). The spliceosome can be isolated from reactions, in the former case with oligo-dT cellulose and in the later case with strepavidin agarose beads. Affinity chromatography after glycerol gradients sedimentation of *in vitro* splicing reactions has shown an association of U2, U4, U5, and U6 snRNAs with the spliceosome. U1 RNA was not seen to be associated under these conditions, in spite of its previously demonstrated requirement in splicing. A more detailed analysis of splicing complexes purified by affinity chromatography under varying conditions has demonstrated the presence of U1 with the pre-mRNA, at least in higher eukaryotic splicing, is sensitive to heparin or high salt concentrations (Bindereif and Green 1987). This finding explains the failure to detect U1 by affinity chromatography in heparin treated spliceosomes isolated from the gradients. The association of the U1 snRNP was shown to be rapid and ATP independent, while that of U2, U4, U5, and U6 was ATP dependent. An assembly order where U1 binding precedes U2 and U4/U5/U6 binding, has been suggested.

Recent experiments using affinity chromatography of yeast splicing reactions also indicate a very early ATP independent binding of U1to the pre-mRNA. U1 binding depends on both the 5' splice site and branch point sequence, and functional U1 binding is a requirement for the subsequent binding of other snRNAs (U2, U4, U5, U6) (S. Ruby and J. Abelson, in press). Using a different approach to address the commitment of pre-mRNA to splicing, Legrain *et al.*, (1988) have shown that an early commitment to splicing takes place in extracts which are depleted in U2 snRNP. This commitment also requires the presence of active 5' splice site and UACUAAC sequence.

The snRNP-mediated recognition events in splicing and in the assembly of the mature spliceosome range from direct base pairing interactions between the snRNA compo-

nents of the snRNPs and the intron sequences, to interactions co-ordinated solely by protein factors. The existence of these protein factors has been demonstrated by the need for micrococcal nuclease- resistant factors in *in vitro* splicing and upon purification of splicing factors (Krainer and Maniatis 1985 ; Cheng and Abelson 1986 ; Kraemer *et al.*, 1987). At least one factor identified from HeLa extracts, U2AF (U2 Auxillary factor) is required for binding of U2 to the substrate. U2AF recognizes the 3' splice site, the polypyrimidine / AG and is required for the formation of a stable pre-mRNA-U2 containing complex (Ruskin *et al.*, 1988). Another factor, the intron binding protein, that also binds the 3' splice site is known. Initial experiments suggest that this protein mediates U5 snRNP interaction with the 3' splice site (Tazi *et al.*, 1986). The suggestion has been made that multiple factors interact independently with the 3' splice site during spliceosome assembly in mammalian systems. Identification of several protein factors required in yeast splicing reactions has been possible through the use of the yeast *rna* mutants, and will be discussed in the next section of this review.

snRNP-snRNP interactions that mediate the formation of higher order structure are still poorly understood. Non-denaturing gels have also been used to detect multiple snRNP interactions that take place in the the extract. In fact, complexes containing the snRNAs have provided one line of evidence for the existence of snRNP complexes in yeast. In both yeast and mammalian systems, U4/U5/U6 snRNA are found to co-exist as a large particle (Konarska and Sharp 1987 ; Cheng and Abelson 1987). In yeast this large particle dissociates into U5 and U4/U6 complexes upon addition of ATP. During the splicing reactions, U4 has been shown to leave the spliceosome before U6 and before the first splicing reaction takes place. Thus the process of assembly and disassembly of snRNPs seems to be a dynamic process. The U1 and U2 RNA of yeast are also associated with proteins as U1 snRNP and U2 snRNP. However they are not associated in any larger multiple snRNP particle.

Purification of mammalian spliceosomes after preparative gel filtration has enabled visualisation of the spliceosomes by electron microscopy. The spliceosome fractions revealed a population of 40-60nm particles (Reed *et al.*, 1988). Yeast spliceosomes partially purified by differential gradient sedimentation have been identified by EM. Ovoid particles of about 20 x 23.5 nm were seen, following tagging with several probes (M. Clark, S.Goelz and J.Abelson, manuscript submitted) .

THE *RNA* MUTANTS OF YEAST

Along with the analysis of snRNA, an important aspect in the genetic analysis of splicing in yeast has been the availability of several trans-acting mutants that affect pre-mRNA splicing. These mutants were identified as temperature sensitive (ts) lethals in the genes *RNA 2* through *RNA11* in a general search for mutants affecting macromolecular biosynthesis in a bank of ts lethals (Hartwell 1967) . Many of these mutants turned out to be involved in splicing because of a peculiarity of the yeast genome. The *rna2-11* mutants showed a rapid cessation of net RNA accumulation upon shifting cultures to nonpermissive temperature. The mutants were subsequently shown to be defective in rRNA synthesis but not in tRNA or mRNA synthesis. The defect in rRNA synthesis was not in transcription but in the processing of the large pre-rRNA (Warner and Udem 1972). Interestingly, these mutations severely decreased the levels of most ribosomal protein mRNAs, and most non-ribosomal protein mRNAs were not affected. Subsequently a number of laboratories found that the genes coding for most ribosomal proteins contain introns. Intron-containing ribosomal transcripts accumulate at non- permissive temperature in these strains, while non intron-containing transcripts were unaffected (Rosbash *et al.*, 1981). These data finally suggested that the defects in rRNA processing were due to defects in the splicing of precursors to ribosomal protein mRNAs. The apparent specificity of the defect was due to the preponderance of introns in genes coding for ribosomal proteins and the rarity of introns in non-ribosomal protein genes. Accumulation of pre-mRNA in other intron-containing yeast

transcripts for *e.g.* actin has also been observed (Teem *et al.*, 1983). Although the deficiency in splicing of the *rna* mutants does not prove that they encode a part of the pre-mRNA splicing apparatus, these mutants were the best candidates for the genetic analysis of the components of the yeast nuclear splicing machinery (Warner 1987).

Evidence for the direct participation of many of the RNA 2-11 gene products in the splicing reaction came from *in vitro* biochemical work (Lustig *et al.*, 1986). This was made possible by the use of a heat inactivation assay with the *in vitro* splicing system. Splicing activity in extracts from *rna* strains are heat sensitive *in vitro* . Seven of the nine complementation groups tested were heat inactivated. This loss of activity in different *rna* mutants was due to the inactivation of an exchangeable component as shown by *in vitro* complementation of pairs of inactivated extracts.

The RNA 3, 5, 7, 8, and 11 gene products are required early in the splicing pathway (Lin *et al.*, 1987) as heat inactivated extracts do not form splicesomes (Fig 3). The *rna 4* extract does form an early splicing assembly intermediate (complex B, Fig 3) after heat inactivation (J. Banroques and J. Abelson, unpublished results). The reason for involvement of many of the RNA gene products early in the splicing pathway is not understood. One reason could be that a large number of factors are required early in spliceosome assembly, or that there are shared components between the different steps of splicing. The RNA2 gene product acts after spliceosome assembly, as heat inactivated *rna2* extracts accumulate spliceosomes (Lin *et al.*, 1987 ; Cheng and Abelson 1987). In the splicing pathway this splicing complex immediately preceeds the reactive spliceosome (AI, Fig1b and Fig 3). Spliceosomes formed in heat inactivated *rna2* extracts are active intermediates in the splicing pathway, and, when isolated from glycerol gradients, the accumulated pre-mRNA can be chased to products. These experiments indicated that RNA2 is an extrinsic component of the spliceosome. These experiments also support the presence of at least two other sets of extrinsic factors, b_n and c_n, required for the first and second reactions of splicing,

respectively. Factor b_n has been shown to be a micrococcal nuclease resistant, heat stable protein(s) required with RNA2 for the first step of splicing.

Several of these *RNA* genes (*RNA* 2, 3, 4, 5, 8 and 11) have been cloned and their molecular characterisation has been initiated. The characteristics are summarised in Table 1. The role that these genes play in pre-mRNA splicing is being investigated by the use of antibodies directed against the proteins. The antisera raised against *RNA2* , *RNA3* and *RNA11* products have been used to localize these proteins to the nuclei (Last and Woolford 1986 ; Chang *et al.*, 1988). In the case of RNA11, immunoelectron microscopy has placed the protein in the periphery of the yeast nucleus.

Antibodies against RNA8-ß gal fusions have identified the protein as an exceptionally large 260 Kilodalton (kD) protein . A role for RNA8 in the splicing pathway has been shown, with experiments demonstrating loss of splicing activity after immunodepletion of RNA8 (Jackson *et al.*, 1988). The RNA8 antibodies have been used to probe for a stable association of this protein with the yeast snRNAs. These antibodies can specifically precipitate U5 from yeast extracts indicating an association of the RNA8 protein and U5snRNA (Lossky *et al.*, 1987). Preincubation of extracts with ATP resulted in immunoprecipitation of U6 and U4 along with U5. The co-precipitation of U4/U6 under these conditions may result from snRNP-snRNP interactions.

Recent experiments with RNA4 antibodies reinforce the view of a direct role in splicing for the RNA4 protein. There are two principal findings. First the RNA4 antisera in immunoprecipitation experiments precipitate yeast U5, U4 and U6 snRNAs. U6 snRNA is known to exist in a complex of proteins as U6 snRNP and also in a particle with U4 snRNA in it. Thus, the co-precipitation of U4 and U6 is not unexpected. Second the RNA4 antibodies inhibit *in vitro* splicing reactions completely. These two results are corroboratory : inhibition of splicing occurs after U2 binds the pre-mRNA (complex B, Fig 3) because anti RNA4 antibodies precipitate U4/U5/U6 snRNAs (J. Banroques and J. Abelson, unpublished observations).

In the case of RNA11, cloned DNA has been transcribed and translated *in vitro* to generate a ^{35}S labeled RNA11 protein (Chang *et al.*, 1988). This labeled protein complements *in vitro* the heat sensitivity of *rna11* extracts. The complementation using the labeled protein has allowed tracing of *RNA11* in splicing complexes. In extracts prepared from *rna11* strain most of the protein enters a 30S complex whose formation is not dependent on the presence of pre-mRNA. A fraction of the labeled protein does associate with 40S spliceosomes; this association is dependent on the presence of splicing signals in the pre-mRNA. Interestingly, the protein sequence is predicted to have a Zn finger domain, previously shown to be a motif found in DNA and RNA binding proteins.

Interactions between the RNA gene products have been indicated both by characterization of extragenic supressors and by experiments showing that increased dosage of one RNA gene product can rescue the lethality of another. The first extragenic suppressor of *rna* mutants to be isolated was *SRN1* (Pearson *et al.*, 1982). Mutants in SRN1 were isolated on the basis of supressing a *rna2 rna6* double mutant . However, SRN1 does not display any allele specificity in its supression, as supression of several *rna* mutants was observed. These results indicate that the different mutants are related but does not indicate any specific interaction between the RNA gene products. Last *et al.* (1987) have defined a specific interaction between RNA3 and RNA4, as extra plasmid-borne copies of RNA3 can rescue the ts lethality of three alleles of *rna4* . The supression of *rna4* requires the presence of the mutant *rna4* protein, indicating that RNA4 is not being bypassed. An extragenic supressor of *rna2* (SRN2) has also been obtained. SRN2 supressed six different alleles of *rna2* but not other *rna* loci. SRN2 is itself an essential gene. The mode of action of SRN2 and RNA3 in supression of *rna2* or *rna4* respectively can be explained by different models. For example : SRN2 and *rna 2* are interacting proteins, *RNA3* and *RNA4* interact, and the overproduction of one allows stabilization of the other by virtue of the interaction .

That the RNA3 and RNA4 gene products interact is also suggested by *in vitro* heat inactivation and complementation data where a weaker complementation than that obtained for other combinations was found (Lustig *et al.*, 1986). These results suggest that they could be part of the same macromolecular complex, a hypothesis consistent with the evidence for the interaction between these gene products.

ISOLATION OF NEW MUTANTS AFFECTING PRE-mRNA PROCESSING

As is evident from the discussions of the *rna2-11* mutants, the use of such an approach to isolation of gene products that play a role in the splicing in yeast has been tremendously fruitful. It is possible to isolate more mutants defective in pre-mRNA splicing. One approach is the generation of temperature or cold-sensitive banks of mutagenised strains, which can be screened for pre-mRNA processing defects. Analysis of conditional phenotypes other than temperature sensitivity would be useful to isolate mutants in some genes that do not readily mutate to give ts alleles.

Our effort to isolate new ts lethals, defining genes involved in pre-mRNA has resulted in the identification of several new complementation groups (U. Vijayraghavan and J. Abelson, manuscript in preparation). Five new complementation groups with three novel phenotypes not seen in the *rna2* to *rna11* mutants were isolated: in one case accumulation of lariat intermediate of splicing was observed and in another case accumulation of the released intron was seen, and in the third case an accumulation of both pre-mRNA and intron was observed. The isolation of a ts mutations causing accumulation of the lariat intermediate provides a unique opportunity to study the components needed for the second step of splicing, cleavage at the 3' splice site and the ligation of exons. *In vitro* splicing using extracts prepared from one of these mutant strains can be heat inactivated, and inactivation results in accumulation of the lariat intermediate and exon1. Intact snRNPs are not required for complementation as micrococcal nuclease treated extracts complement inacti-

vated extracts. Spliceosomes formed after heat inactivation contain an expected lariat intermediate and exon1. These intermediates can be chased to products upon addition of complementing fractions to the assembled mutant spliceosome. These chasing experiments have also defined a requirement for ATP in the second step of the splicing reaction.

The accumulation of the intron in one complementation group does not confer a temperature sensitive phenotype, and the isolation of the mutant as a ts was serendipitous. The cellular intron in this mutant exists in a large particle of about 40S in extracts prepared from mutant strains. Co-sedimentation of snRNPs with the intron-containing particle suggests an association of the intron with the snRNPs. Tri-methyl G cap and Sm specific antibodies that precipitate snRNPs, also precipitate intron from whole cell extracts, supporting the view that the intron is associated with snRNPs. The accumulation of the intron could be due to the defect in a lariat debranching function, or due to a defect in the disassembly of the intron-containing particle after splicing has been completed.

A different approach to isolation of mutants in gene products involved in splicing is applicable to yeast for the study of macromolecular complexes. This is the isolation of extragenic supressors to ts mutants in the *rna2-11* groups. These supressors could define interacting gene products, which are themselves a part of the splicing machinery. In such an approach several pseudo-revertants to *rna4* that allow growth at a temperature that is non-permissive have been obtained. These pseudo-revertants (*srn4*) themselves confer a cold-sensitive phenotype to the cell and segregate as single nuclear recessive genes (J. Maddock and J. Woolford, per. comm.). None of these pseudo-revertants of *rna 4* are allelic to known *rna* genes. *srn4-1,* and *srn4-2* supress *rna4* on the basis of their isolation and also supress *rna3* . RNA4 and RNA3 have previously been shown to interact and the isolation of a supressor to both is not surprising. The supressors do not work by substituting for the RNA4 or the RNA3 function as supression by *srn4* does not work with null alleles of RNA4 or RNA3. *srn4-1* and *srn4-2* are candidates for mutations in genes whose products directly interact with these RNA genes. The use of cold-sensitive supressors of ts

mutations should bring the system around to a complete circle genetically, with mutants in genes for some components supressing ts mutants in genes for other components. Such analysis would also be extremely useful to map out interactions between these gene products.

A parallel genetic approach for identifying the components of the splicing machinery is the isolation of trans-acting supressors to point mutants in the pre-mRNA splicing signals that are essential for splicing. A trans-acting supressor, that restores splicing of a branch point mutation in a yeast intron has been isolated (Couto *et al.*, 1987). This allele-specific supressor behaves as a single Mendelian gene, with a dominant supression phenotype. A recessive effect of accumulation of unspliced wild type pre-mRNA was also observed. These two phenotypes argue for a direct role for the *RNA16* locus in the splicing machinery. A similar approach to isolate supressors to point mutations in essential regions of spliceosomal snRNAs, should allow definition of components of snRNPs.

The isolation of trans-acting conditional mutants affecting pre-mRNA splicing, the isolation of supressors to these mutants and the isolation of supressors to mutants in the pre-mRNA should define many gene products of the splicing apparatus. The analysis of the effects of conditional mutants biochemically should be possible when extracts prepared from these strains are inactivated *in vitro*. Inactivation and complementation of these extracts would be useful in delineating the steps in splicing assembly pathway that they affect. The analysis of defects in the assembly itself is now possible using improved methods of separation of splicing complexes on native gels, and the detection of binding of splicing components to pre-mRNA on solid supports.

The assembly of snRNPs in wild type extracts using *in vitro* synthesised snRNAs are now being studied. Once the wild type pattern of assembly is known, study of the effect of inactivated mutant extracts or extracts from supressor strains on the assembly pattern of individual snRNPs and also their effects on multi-snRNP complexes (e.g. U4/U5/U6) can be attempted. Thus, a variety of tools necessary for a concerted biochemical and ge-

netic analysis of pre-mRNA splicing are available, and analyses toward this end are in progress.

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TABLE : 1 MOLECULAR CHARACTERIZATION OF *RNA* GENES

	ts *in vitro*	Required for Spliceosome formation	GENE Cloned	GENE Essential	Gene Products	Antibody Experiments
RNA2	Yes [a]	No [a]	Yes [b, c, d]	Yes [b, c, d]	~2.5 kb Transcript [b] ~100 kD protein	Nuclear [b] protein
RNA3	Yes [a]	Yes [a]	Yes [b]	Yes [b]	~1.5 kb Transcript [b] ~56 kD protein	Nuclear [b] protein
RNA4	Yes [a]	Yes [a]	Yes [d]	Yes [d]	~1.6 kb Transcript [d] ~52kD protein [f]	Inhibit splicing [f] Precipitate U5, U4, U6 snRNA
RNA5	Yes [a]	Yes [a]	Yes [e]	?	~1.4 kb Transcript [e] ~47 kD protein	In progress [e]
RNA6	No [a]	?	No	?	?	?
RNA7	Yes [a]	Yes [a]	No	?	?	?
RNA8	Yes [a]	Yes [a]	Yes [g]	Yes [g]	~7.4 kb Transcript [g] ~260 kD protein	Inhibit splicing [g] Precipitate U5snRNA
RNA9	No [a]	? [a]	No	?	?	?
RNA10/11	Yes [a]	Yes [a]	Yes [b, d]	Yes [b, d]	~1.0 kb Transcript [b] ~30 kD protein [h]	Inhibit splicing [h] Nuclear protein

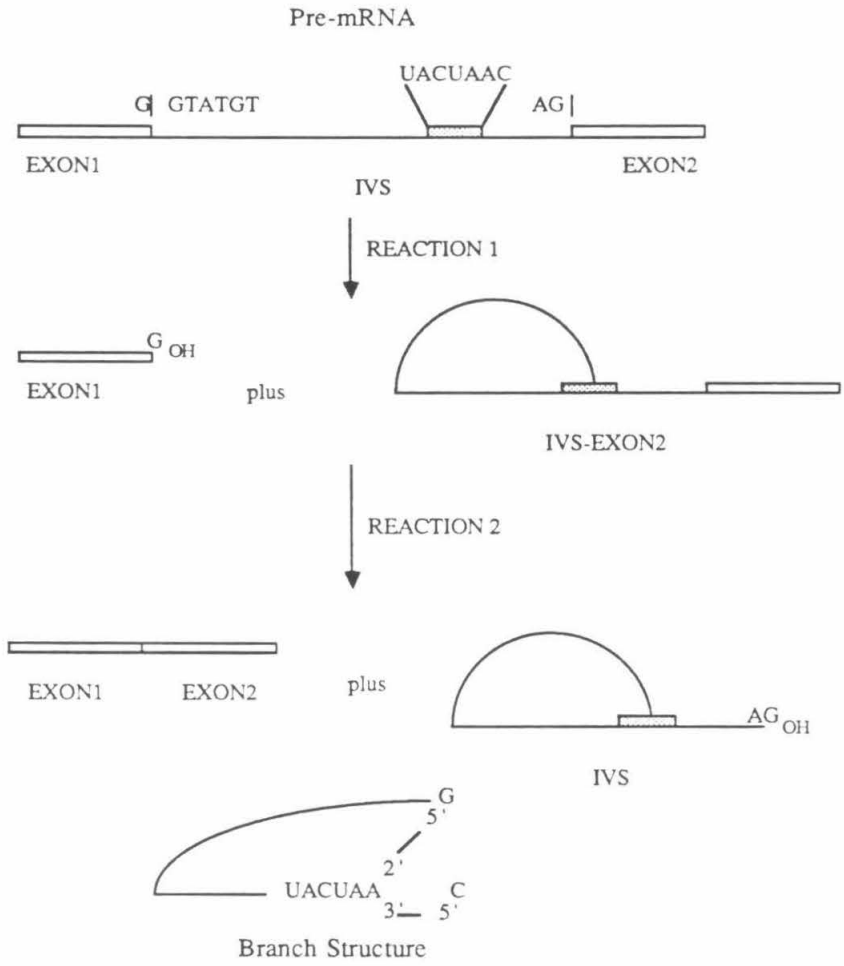
a. Lin et al. 1987 ; Lustig et al. 1986
b. Last et al. 1984 ; Last et al. 1986
c. Lee et al. 1984
d. Soltyk et al. 1984
e. McFarland and Abelson unpublished
f. Banroques and Abelson unpublished
g. Jackson et al. 1988 ; Lossky et al. 1987
h. Chang et al. 1988

FIGURE LEGENDS

Fig 1a. The pathway of pre-mRNA splicing reactions. The reactions are described in the text. The intron-exon junctions are represented by vertical bars. The structure of the yeast branch is shown.

Fig 1b. Scheme for the assembly of the spliceosome. The snRNA content of each splicing complex is indicated. A2-2 represents the active spliceosome.

a


Pre-mRNA
UACUAAC
G GTATGT
AG
EXON1
EXON2
IVS
REACTION 1
G OH
EXON1
plus
IVS-EXON2
REACTION 2
EXON1
EXON2
plus
AG OH
IVS
G
5'
2'
UACUAA
C
3'
5'
Branch Structure

b

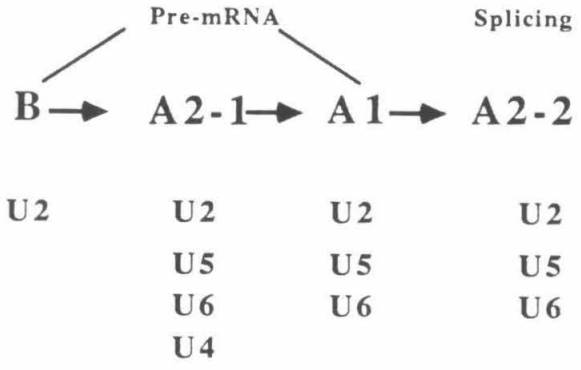

Pre-mRNA
Splicing
B
A2-1
A1
A2-2
U2
U2
U5
U6
U4
U2
U5
U6
U2
U5
U6

Fig 2. Representation of pre-mRNA and snRNA interactions. Base pairing of U1 and U2snRNPs with the 5' splice site and the branch sequence respectively has been demonstrated genetically (see text). Binding of U5 snRNP is proposed to be mediated by a protein in mammalian systems. The U4 and U6 binding sites to the pre-mRNA are not known. Black circles represent Sm binding sites. [This fig. was contributed by P. Siliciano and C. Guthrie]

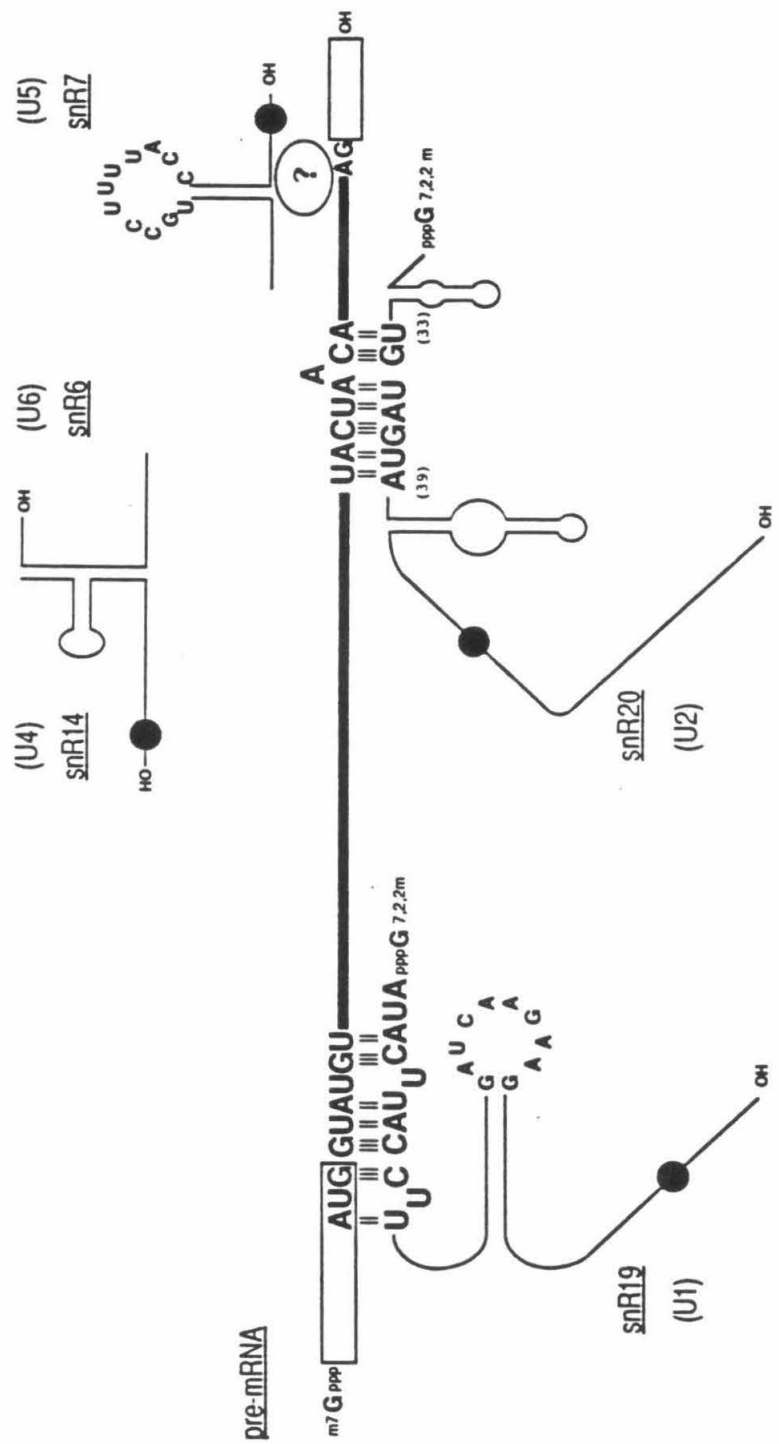
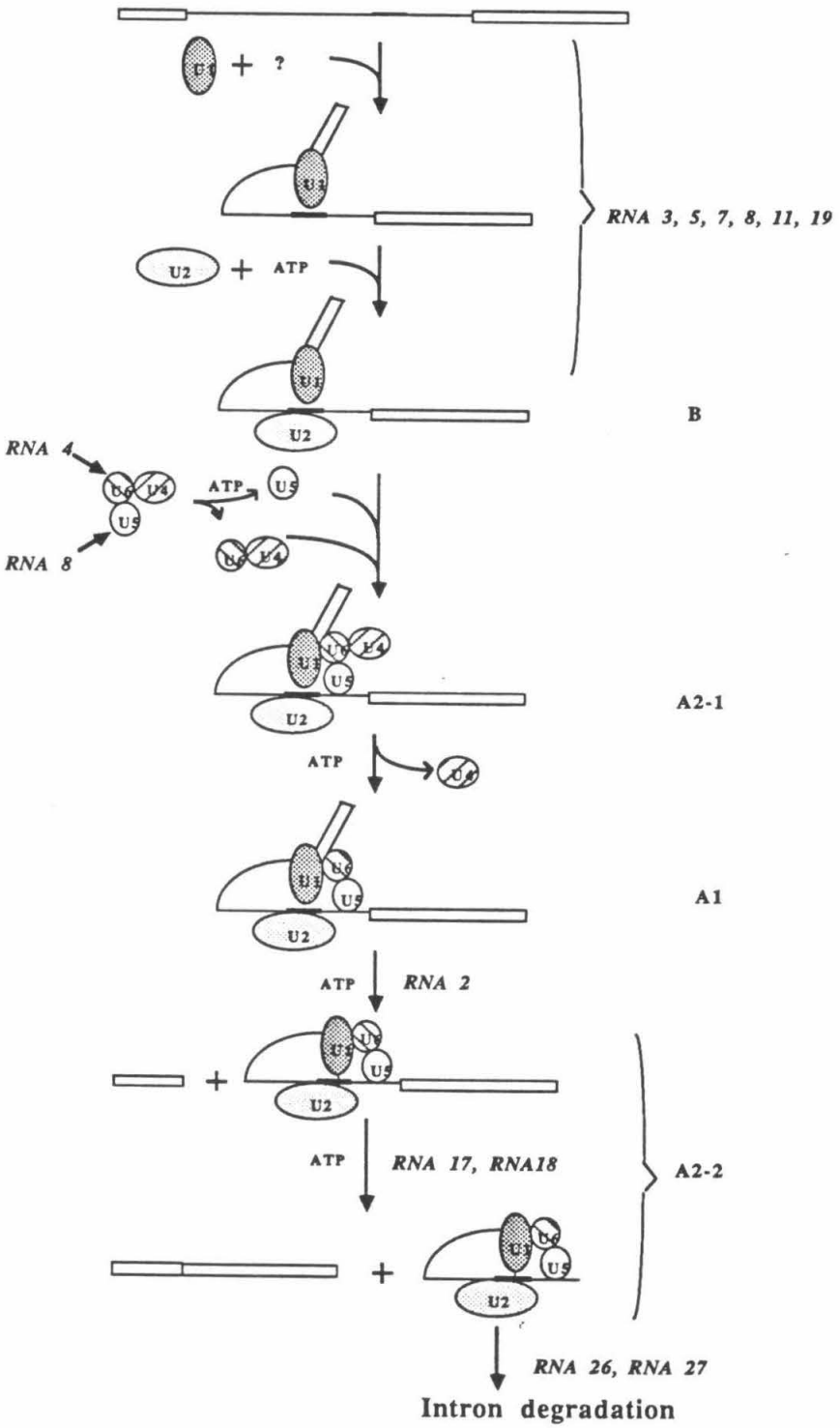

pre-mRNA
m7G ppp
AUG
GUAUGU
U C CAU CAUA pppG 7.2.2m
U
snR19
(U1)
snR20
(U2)
(U4)
snR14
(U6)
snR6
(U5)
snR7
UACUA CA
AUGAU GU
(39)
(33)
pppG 7.2.2 m
OH
HO
?

Fig 3. Proposed pathway for the assembly of the spliceosome. The role of the *RNA* gene products in the assembly is indicated where known.


U1
+ ?
RNA 3, 5, 7, 8, 11, 19
U2
+ ATP
U1
U2
B
RNA 4
U6
U4
ATP
U5
U5
RNA 8
U6
U4
U1
U6
U4
U5
U2
A2-1
ATP
U4
U1
U6
U5
U2
A1
ATP
RNA 2
U1
U6
U5
U2
+
ATP
RNA 17, RNA18
A2-2
U1
U6
U5
U2
+
RNA 26, RNA 27
Intron degradation

CHAPTER 1

Mutations in Conserved Intron Sequences Affect Multiple Steps in the Yeast Splicing Pathway, Particularly Assembly of the Spliceosome

Key words: Site-directed mutagenesis / yeast splicing pathway / spliceosome

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ABSTRACT

Yeast introns contain three highly conserved sequences, which are known to be required for splicing of pre-mRNA. Using in vitro mutagenesis, we have synthesized seven point mutations at five different sites in these signals in the yeast actin intron. The mutant introns were then inserted into each of three constructs, which allowed us to assess the consequences on splicing both *in vivo* and *in vitro* . In virtually every case, we found the efficiency of splicing to be significantly depressed; mature mRNA levels *in vivo* ranged from 0 to 47% of wild-type. Surprisingly, the tightest mutations were not necessarily at the sites of nucleolytic cleavage and branch formation; these nucleotides are thus highly preferred, but are not absolutely necessary. Moreover, while particular nucleotides are specifically required for the final step in splicing, i.e., 3' cleavage and exon ligation, the predominant consequence of mutation within the conserved signals appears to be the inhibition of assembly of the splicing complex.

INTRODUCTION

The coding sequences of the majority of eukaryotic genes are interrupted by introns. The removal of intervening sequences by the process of RNA splicing is thus a fundamentally important step in gene expression, yet one which is only poorly understood at the molecular level. The most enigmatic aspect of this class of reactions is how the splice junctions are identified and juxtaposed, despite their separation in the primary structure by as many as 100,000 nucleotides (Scott *et al* ., 1983; Garber *et al* ., 1983).

Progress toward an understanding of this problem has been most rapid for the so-called group I introns, which comprise the intervening sequences from nuclear and mitochondrial rRNA, many mitrochondrial mRNAs, and several chloroplast tRNA precursors (Michel *et al* ., 1982; Davies *et al* ., 1982; Michel and Dujon, 1983; Waring *et al* ., 1983; Cech *et al* ., 1983). These introns share a set of highly conserved structural elements that dictate a specific folding pattern. The folding opposes the intron/exon borders by align-

ment against an "internal guide sequence." In fact, the three-dimensional geometry of this structure plays an even greater role, in that several of these precursors have been shown to undergo autocatalytic splicing in vitro in the complete absence of protein (Kruger *et al* ., 1982; Garriga and Lambowitz, 1984; Van der Horst and Tabak, 1985).

This situation contrasts sharply with the problem of splice site selection in metazoan nuclear mRNA precursors. Here the only conserved elements are restricted to short sequences at the 5' and 3' junctions (Mount, 1982). Even so, there is considerable variation about these averages, and more problematically yet, these sequences do not appear to be absolutely required for splicing, in that both cryptic and normal junctions are known that fail to obey even the 5' dinucleotide consensus (see, e.g., Mount and Steitz, 1983). A considerably clearer picture has emerged from yeast, where analyses of intron sequences have revealed three highly conserved elements required for splicing. These are the 5' consensus sequence GTAPyGT, the 3' junction sequence PyAG, and the sequence TACTAAC that lies near the 3' junction and functions as the branch point (Domdey *et al* ., 1984, Rodriguez *et al* ., 1984). Despite this stronger conservation at the level of primary sequence in yeast splicing signals, the challenge of explaining accurate juxtaposition of splice junctions without the assistance of intron-mediated folding applies equally to lower and higher eukaryotes.

The missing pieces of the splice junction puzzle are likely to be found among the molecular components of the splicing machinery. Lerner and Steitz (1979) and Rogers and Wall (1980) first suggested that 5' and 3' junctions could be aligned via complementarity between these sequences and a stretch of 18 nucleotides at the 5' end of the U1 snRNA. While the U1 snRNP binds to the 5' splice site (Mount *et al* ., 1983), evidence does not support base pairing between the 5' end of U1 and the 3' splice site (Mount and Steitz, 1981; Mount *et al* ., 1983). Rather, it appears that the 3' region of the intron provides binding sites for two snRNPs; the U2 snRNP binds and protects the branchpoint (Krainer and Maniatis, 1985; Black *et al* ., 1985), and U5 snRNP, probably,the 3' splice site

(Chabot *et al* ., 1985). From these results, a reasonable conjecture is that the splice sites are positioned via the interactions of the snRNPs with one another. Consistent with this notion is the observation that splicing intermediates in yeast (Brody and Abelson, 1985), as well as in HeLa (Grabowski *et al* ., 1985), are associated with a rapidly sedimenting, presumably multicomponent complex, named the spliceosome.

A more detailed understanding of the relationship between splicing signals and the splicing machinery would benefit from the combined approaches of genetics and biochemistry. Toward this end, we have recently reported the use of gene fusions for the identification and characterization of splicing mutations in *Saccharomyces cerevisiae* (Parker and Guthrie, 1985; Cellini *et al* ., 1986). Since the yeast splicing machinery, in contrast to that in mammals, does not use cryptic junctions (Gallwitz 1982; Pikielny *et al*., 1983; Newman *et al* ., 1985), it has been possible to identify the molecular consequences of mutant signals. For example, we have previously shown (Parker and Guthrie, 1985) that mutation of the fifth nucleotide of the 5' consensus sequence results in pleiotropic effects that uncouple three steps in splicing: 1) initial recognition of the 5' signal, 2) 5' cleavage and branch formation, and 3) 3' cleavage and ligation (as a consequence of aberrant cutting at an upstream site).

Here we report an extension of this strategy for the systematic analysis of the effects of mutations within each of the three conserved signals in the yeast actin intron. Our results indicate that while certain residues are required specifically for the last step in splicing, 3' cleavage and exon ligation, the predominant consequence of mutation within the conserved signals is the inhibition of assembly of the splicing complex. These findings are consistent with the view that the strength of interaction between *cis* -acting signals in the substrate and *trans* -acting recognition factors, is likely to include snRNPs.

RESULTS

Mutant construction

Using oligonucleotide directed mutagenesis (Newman *et al* ., 1983), we first generated a number of point mutations within the intron of the yeast actin gene (Ng and Abelson, 1980). In order to monitor the consequences of these mutations both *in vivo* and *in vitro,* the mutant introns were then introduced into each of three constructs, as described in Figure 1. The first construct (pYAH-I2) is a fusion between the actin intron and the yeast *HIS*4 gene. The characteristics of this chimeric gene have been described previously (Parker and Guthrie, 1985). Briefly, accurate and efficient splicing of the actin intron allows *HIS*4 deletion strains to grow on media containing the histidine precursor, histidinol (Hol; see Experimental Procedures). In addition, we can characterize the resultant transcripts by using the biochemical methods described below. The ultimate appeal of this construction is in future studies, in which we can select suppressors of the original splicing defects by demanding growth on Hol.

The second construct (pYAHB-2) utilizes a fusion to the *E. coli* beta-galactosidase gene. As we have shown (Larson *et al* ., 1983; Cellini *et al* ., 1986), this construction allows a simple and rapid quantitative assay for the efficiency of splicing. Finally, to enable analysis of the intron mutants in vitro, an AluI fragment from the actin *HIS*4 fusion was transferred into an SP6 transcription vector. This allowed the convenient synthesis of mutant precursors for subsequent analysis in the yeast splicing system recently described (Lin *et al* .,1985).

The yeast actin intron contains the sequence TACTAAG, just upstream of the TACTAAC box. We have previously reported that this sequence can function as a branch point in the absence of the authentic TACTAAC box (Cellini *et al* ., 1986). Moreover, the first mutation we analyzed in the TACTAAC box (A256, see below, Figure 4), activated this sequence as a branchpoint. To avoid the potential complication of this contribution in our further analyses, we deleted six nucleotides comprising this cryptic TACTAAC box

prior to the construction of additional mutants (see Figure 1). This deletion, referred to as Δ6, exhibits wild-type activity in all our splicing assays both *in vivo* and *in vitro* .

In selecting our targets for site-directed mutagenesis, we were guided by the rationale that evolutionary conservation is indicative of a functionally important role. As we have discussed recently (see Guthrie *et al* ., 1986), the virtually 100% conservation at every position within the splicing signals of *Saccharomyces cerevisiae* introns is rather non-illuminating in this regard. In contrast, comparison of intron sequences from other fungi reveals more substantial variation within these signals (cf. Figure 6, Guthrie *et al* ., 1986) and thus allows the identification of particularly conserved nucleotides. Based on these arguments, we constructed the mutations depicted in Figure 1. Mutations are designated as the nucleotide introduced and the position of the change within the actin intron, which is numbered from the 5' junction (/G is position 1) to the 3' junction (G/ is position 303. These include: 1) alteration of the first base of the intron from G to A or C (referred to as A1 and C1, respectively); 2) four mutations at three positions in the TACTAAC box, A256 and T256, A257, and C259; and 3) alteration of the 3' splice site from CAG to CAC. (Because the dinucleotide immediately following the wild-type 3' junction is also AG, we constructed the double mutant, C303/305, changing the sequence at the 3' splice junction from CAG/AG to CAC/AC. In this construction, the next AG is 44 nucleotides downstream.) In addition, since the exon base next to the 5' junction is a G in approximately 70% of all spliced genes (Mount 1982; Guthrie *et al* ., 1986), we altered this nucleotide to a C, designated C0. Finally, we have included the A5 mutation that we previously characterized *in vivo* (Parker and Guthrie, 1985).

In vivo characterization

Biological phenotypes

As an inital assay for the level of splicing we determined the amount of β-galactosidase activity produced from the various mutations in the centromere plasmid pYAHB2 depicted in

Figure 1. The plasmid was introduced into the yeast strain NNY1 by transformation and Trp^+ transformants were assayed for β-galactosidase activity in liquid culture. With the exception of C0 and T256, all mutations result in a significant reduction in enzymatic activity and therefore presumably inhibit splicing (see Table 1). Transformants containing the A256 mutation exhibit the highest level of residual activity, about 47% activity relative to wild-type. Note that the level of β galactosidase expression for the A256 mutation in the Δ6 intron is significantly lower than that seen when the same mutation is present in a wild type intron; the significance of this will be discussed later. As indicated in Table 1, mutants in which residual splicing is between 30% and 50% exhibit a leaky Hol^+ phenotype.

Transcript analysis

The results of the β-galactosidase assays suggested that most of the mutations produced major effects on splicing. As direct confirmation, we next analyzed the transcripts produced *in vivo* by the method of primer extension. For these experiments, yeast strain FC8-24D was transformed with the various mutant forms of pYAH-I2 (see Figure 1). $Poly(A)^+$ RNAs were prepared and used as templates in primer extension experiments, as detailed in Materials and Methods .

Use of a primer located in the 3' exon allows us to examine the ratio of mature to precursor RNA and to determine the levels of lariat RNA molecules in the cell; note that this primer is derived from *HIS*4 sequences and is thus specific to the fusion in these cells (see schematic to Figure 2). As shown in Figure 2, in both wild type (lane 3) and delta 6 constructs (lane 4), only mature RNA (Band 2) is visible, indicating that the delta 6 deletion does not alter the efficiency of splicing *in vivo*. Consistent with the results from the lacZ fusion plasmids in Table 1, all mutations within the intron, with the exception of T256, result in strongly decreased levels of mature fusion mRNA.

The A1 and C1 mutations, which correspond to alterations of the same nucleotide, produce unexpectedly different results. In the case of A1 (Figure 2, lane 7), the predomi-

nant consequence is the accumulation of lariat molecules (Band 3), while there is a slight increase in the level of full-length precursor RNA (Band 1). This lariat RNA must be a dead-end product, since no mature mRNA is produced. In contrast, the C1 mutation (lane 6) results in a barely detectable amount of lariat, while the major accumulated species is full-length precursor; as with A1, no mature RNA can be detected. As observed previously (Parker and Guthrie, 1985), the A5 mutation causes the accumulation of full-length precursor as well as some lariat species; mature mRNA production is decreased relative to wild-type.

Three mutations in the TACTAAC box, A256 (cf. lane 4, Figure 3; lane 6, Figure 4), A257 (lane 10, Figure 2), and C259 (lane 11, Figure 2) cause a dramatic accumulation of precursor molecules. The finding that C259 does, however, produce some mRNA is interesting, since it is the branch point nucleotide that is altered. In fact, the A257 mutation, which is two nucleotides 5' to the site of branch formation, produces a stronger inhibition of production of mature message. These differences are also reflected in the β-galactosidase levels (cf. Table 1).

The C303/C305 mutation is unique in that it appears to affect only the last step of the splicing pathway. As seen in Figure 2, lane 12, there is no detectable full-length precursor, suggesting that 5' cleavage and lariat formation takes place efficiently, while there is a dramatic accumulation of molecules with the mobility of lariats, consistent with a block in 3' cleavage and ligation. It is interesting to note, however, that a low but detectable level of mRNA-sized molecules is found, despite the presence of a C at the 3' cleavage site. (Evidence that cleavage is occurring at this residue, i.e., AC/, is presented below).

The interpretation of each of the preceding results was confirmed by additional primer extension experiments. As shown in Figure 3, the use of an oligonucleotide primer situated 46 nucleotides downstream of the 5' splice junction reveals the accumulation of molecules indicative of full-length precursor for mutants C1 (lane 1), A1 (lane 2), A5 (lane 3) A256 (lane 4), C259 (lane 5) and A257 (lane 8). We also observe the accumulation of

presumptive lariat species in the case of A1, C303/305, and to a much lesser extent, A256 (lane 4). In the latter cases we can thus also conclude that the position of the 5' cleavage is unaltered as the result of mutation at the indicated positions. As we have reported previously (Parker and Guthrie, 1985), this is not the case when the fifth position of the 5' junction is altered; the A5 mutation activates an aberrant cleavage site upstream of the normal splice site (see Figure 3, lane 3).

To confirm that the primer extension block at the TACTAAC box assigned to the lariat species is indeed due to a branched species, we made use of an enzymatic activity that hydrolyzes 2'-5' phosphodiester bonds (Ruskin and Green, 1985). Treatment of branched molecules with this "debranchase" has been shown to remove the block to reverse transcription and allow the enzyme to extend to the 5' terminus of the RNA template, in this case the 5' intron/exon junction. In the experiment shown in Figure 4, we used a primer that is located near the branch site in order to facilitate precise mapping of the branchpoint. As can be seen most clearly for C303/305, the band corresponding to the lariat block (band 3, lane 12) is almost completely abolished following prior incubation with debranchase (lane 11). Note the concomitant appearance of a novel species with the appropriate mobility for molecules cleaved at the 5' junction (lane 11, band 2).

Comparable results are seen for A1 (Figure 4, lanes 15, 16) and C259 (Figure 4, lanes 7, 8). The latter finding is particularly important, as we noted above, because the C259 mutation alters the branch point nucleotide. The fact that the branch still forms at the correct position demonstrates that the chemistry of branch formation is not limited to a 2'-5 phosphodiester linkage between G1 and an A residue. Apparantly a 5'G-2'C linkage can also form, albeit inefficiently. This result is in interesting contrast to the situation in A256 when this mutation is present in an otherwise wild-type (i.e., non- Δ6) intron background. A debranchase-sensitive stop is apparent not only at the normal position (Figure 4, Band 3, lane 5 cf. lane 6), but also at the upstream cryptic sequence (Figure 4, Band 4); the latter primer extension stop maps precisely to the last A of the cryptic TACTAAG box. Thus de-

spite the presence of the normal branch point nucleotide, the A256 mutation activates the use of the cryptic site (see Cellini *et al* ., 1986). That this is a productive branchpoint is indicated by the observation that β-galactosidase levels are reproducibly higher in strains carrying the cryptic element as compared to those in a Δ6 background (cf. Table 1).

In vitro analysis

The results of the preceding analyses demonstrate that, with the exception of T256, each of the intron mutations we constructed had drastic consequences on the efficiency of the splicing reaction *in vivo* . Moreover, with the exception of C303/305, the defect was primarily manifested by the accumulation of full-length precursor molecules. We next sought to confirm these results *in vitro* . Two additional pieces of information were also of interest. First, in that the *in vivo* data are perforce restricted to steady state analyses, we wanted to determine whether additional effects could be discerned by kinetic analyses. Second, we were particularly curious to learn if the accumulation of precursor molecules could be more specifically attributed to one of two possible blocks, 5' cleavage *per se*, or the earlier event of spliceosome assembly.

In vitro splicing assay

We first assayed the ability of the the mutant substrates to be spliced *in vitro*. In these experiments, radioactive precursor generated by *in vitro* transcription with SP6 polymerase was incubated for various times in an active splicing extract, and the products of the reaction were separated by polyacrylamide gel electrophoresis. Figure 5 shows the now familiar products of splicing that are obtained with wild-type or Δ6 actin precursor. The ligated mRNA product (Figure 5a, band 5) can be seen to accumulate with time. The excised intron is released as a lariat (Figure 5a, band 4), which characteristically (Ruskin *et al* ., 1984; Domdey *et al* ., 1984) migrates more slowly than the precursor (Band 1). Two intermediates are seen, the free 5' exon (Figure 5a,band 3), and the most slowly migrating

form, the intron-exon 2 lariat (Figure 5a, band 2). The same results are seen for the CO mutation (Figure 5d), confirming that this change has no detectable effect on recognition of the 5' splice site and its utilization for splicing.

The three 5' splice site mutants, A1 and C1 (Figure 5b and c) and A5 (data not shown), are completely inactive *in vitro* ; only full-length precursor is detected, indicating that the block has occurred at a step prior to the first nucleolytic event of splicing. The same result is found for the mutations in the TACTAAC sequence, A256 and A257 (Figure 5e and 5f) and C259 (data not shown). In that the C259 mutation is detectably leaky *in vivo* (7% by β-galactosidase assay), we attempted to force the *in vitro* analysis by using substantially nuclease free extracts thereby permitting prolonged incubations with precursor RNA. As shown in the last panel of Figure 5, under these conditions, which entail use of a 35% ammonium sulfate precipitation of the whole cell extract, small amounts of intron lariat can be observed during long periods of incubation. We attribute the failure to detect the production of IVS-exon2 to insufficient sensitivity, in that this species is typically found at about 25% the level of the excised intron if utilization of the lariat intermediate in the second nucleolytic step is efficient.

In the 3' splice site mutant C303/C305, the *in vitro* results again mimic our findings *in vivo* . As shown in Figure 6(a), lariat intermediate and exon 1 accumulate (Band 2), but no mRNA product is formed; thus the second nucleolytic step in splicing is completely inhibited. This 3' splice site mutant was of particular interest in light of the conclusion from our *in vivo* analyses (cf. Table 1 and Figure 2) that some low level cleavage was occurring at the correct position with respect to the 3' junction, despite the change in nucleotide sequence. To provide more direct evidence for this hypothesis, we turned to the 35% ammonium sulfate pellet of the whole cell extract, which generated a small but detectable amount of excised lariat intron (Figure 6b). This product has the same electrophoretic mobility as the wild-type lariat intron and hence cannot result from cleavage at a sequence downstream of the now mutant 3' splice site. To determine the exact site of cleavage, RNase T1 finger-

print analysis of the excised intron was performed. Figure 7a shows the pattern for the Δ6 intron, which contains the oligonucleotide UUUAG-OH derived from the 3' junction (designated as 14' by Lin *et al* ., (1985)). This spot is missing in the T1 fingerprint from the mutant C303/305; as shown in Figure 7b, it is replaced by a spot whose mobility is consistent with a single base change, as expected for the mutation of the terminal G to a C. Consistent with this observation, T_1 oligonucleotide from the intron-exon2 junction ACACG was not seen in the T1 fingerprint of the mutant intron (Figure 7b). To prove the identity of this product, a transcript was prepared by labelling with α-^{32}P CTP and the excised intron was isolated. The putative 3' terminal T1 oligonucleotide UUUAC-OH was incubated with pancreatic ribonuclease A; separation of these products by thin layer chromatography revealed the presence of the predicted labeled product Ap*C-OH (data not shown). We conclude that the mutation of the splice junction from PyAG/ to PyAC/ decreases the efficiency but not the fidelity of the 3' cleavage reaction.

Spliceosome assembly

As first reported by Brody and Abelson (1985), Figure 8a shows that exogenous labeled precursor (in this case the Δ6 transcript) when incubated with splicing extract, is incorporated into a complex that sediments at 40S in a glycerol gradient. The peak fractions contain full-length precursor, the reaction intermediates and excised intron, but they are deficient in mRNA (Brody and Abelson 1985). Formation of this complex is ATP-dependent; in the absence of ATP, a 30S complex forms (Fig 8a; Brody and Abelson, 1985), which contains unspliced precursor only.

Figure 8b, c, and e-g shows the strikingly uniform finding that the three 5' splice site mutants A1, C1 and A5, and each of the mutations in the TACTAAC sequence, A256 (data not shown), A257 and C259, block formation of the 40S complex; 30S complex is apparently unaffected. These results are in dramatic contrast to the behavior of the 3' splice site mutant C303/305 transcript. As shown in Figure 8h, this precursor assembles as effi-

ciently as does the Δ6 control into a species whose mobility (40S) is indistinguishable from that of the parental complex. This observation is consistent with the demonstration that the C303/305 mutant is capable of undergoing the first nucleolytic event in splicing *in vivo* and *in vitro*, 5' cleavage and branch formation. As shown in Figure 8d, the C0 mutation, which permits efficient splicing *in vivo*, also enables the formation of the spliceosome *in vitro* .

DISCUSSION

Genetic dissection of splicing in vivo

At our current level of understanding, three stages of nuclear pre-mRNA splicing can be operationally identified. As cartooned in Figure 9 for yeast, the process appears to be initiated by an ATP-dependent assembly reaction, which results in the formation of a rapidly sedimenting complex, the spliceosome (Brody and Abelson, 1985; Grabowski *et al* ., 1985; Fredneway and Keller, 1985). Cleavage at the splice junctions and subsequent ligation then proceeds by a two-stage reaction. In the first step, the 5' end of the intron forms an intramolecular branch via a 2'-5' phosphodiester bond with the 3'-most A in the TACTAAC box, creating a lariat structure. In the second step, the 3' exon is joined to the 5' exon, accompanied by the release of the intron lariat. In contrast to the Group I and Group II introns in protozoan rRNA and certain organellar tRNA and mRNA precursors (Michel *et al* ., 1982; Michel and Dujon, 1983), the only *cis* elements that are likely to mediate these events are confined to short sequences at the sites of nucleolytic cleavage and branch formation.

By mutational alteration of each of these three conserved signals in the yeast actin intron, we have sought in a systematic fashion to determine the specific contribution of individual nucleotides to the mechanism of splicing. In the first level of analysis the goal is to dissect the pathway into its component partial reactions and to assess the relationship of these steps to one another. Moreover, the response of the system to mutational perturbation of the substrate should be indicative of fundamentally important aspects of the splicing ma-

chinery. The ultimate potential of a genetic approach would be realized by the use of these mutant signals for the direct identification of mutations in the components that interact with them.

Evolutionary conservation identifies critically important nucleotides in multiple steps in the splicing pathway

Our choice of nucleotide alterations was based on the observation that certain positions within these signals show higher conservation than others, if introns from a variety of filamentous fungi are included in addition to *Saccharomyces cerevisiae* in the sequence comparisons (cf. Guthrie *et al* ., 1986). In keeping with the prediction that evolutionary conservation would identify functionally critical residues, we have found that alteration of each of the five positions tested has severe consequences on splicing efficiency. With one exception (see below), residual mature mRNA levels ranged from undetectable to 47% of wild type, measured by Δ-galactosidase production. Consistent with this prediction, both Jacquier *et al* . (1985) and Fouser and Friesen (1986) have recently shown that mutations in less conserved positions have comparably less dramatic effects on the level of mRNA production.

While alteration of each of the three signals had profound effects on splicing, the consequences of individual mutations were quantitatively and qualitatively distinct. For example, the conservative transition mutation at the third position of the TACTAAC box (T256) has an undetectable effect on the level of mRNA formation, while the C to A transversion at the identical position (A256) reduced mRNA production by 50%. A singularly dramatic example of qualitative differences is provided by considering the effects of mutation of the first base of the intron from G to C (C1) or to A (A1). In the case of C1, only full-length precursor is observed and while precursor levels in A1 are also elevated relative to the parental control, the predominant block is in the conversion of lariat intermediates (Figure 9, Step 4). This suggests that a particular position may participate in more

than one reaction in the splicing pathway, while the specific requirements for the chemistry of these individual reactions may differ considerably. Thus a pyrimidine may not be tolerated in one or more steps leading to 5' cleavage and branch formation (Figure 9, steps 1-3), while an A residue is acceptable until step 4. The apparently absolute requirement in 3' cleavage for a G residue at the 5' position of the branch has been discussed previously (Newman *et al* ., 1985; Parker and Guthrie, 1985).

Site specificity of bond breakage/formation is not dependent on the conserved nucleotides at these postions

The surprising finding that 5' cleavage could occur, albeit inefficiently, in the absence of the completely conserved G at the intron border (Newman *et al* ., 1985; Parker and Guthrie, 1985) can now be extended to 3' cleavage. That is, replacement of the invariant G at the 3' junction by a C residue (C303/305) does allow a low level of splicing via cleavage at this mutant site. Since no downstream sequences are utilized as 3' splice sites, the site specificity for the low level of cleavage at the mutant site could be conferred by the distance from the TACTAAC sequence or the sequence context around this mutant site. Moreover, we have shown that replacement by a C (C259) of the absolutely conserved A residue, which provides the 2' hydroxyl in the generation of the lariat, permits unexpectedly efficient branch formation at the mutant residue. This forces us to conclude that, their extraordinary evolutionary conservation notwithstanding, the specific residues at the sites of nucleolytic cleavage and branch formation are highly preferred, but not absolutely required. Indeed, the contribution of these nucleotides is most apparent in modulating the efficiency of the reactions in which they participate. The significance of this observation is underscored by our previous demonstration that, at least for the case of 5' cleavage, the specificity of the reaction, while unaffected by the mutation at the site of bond breakage (A1, C1), can nonetheless be altered in response to mutation elsewhere in the signal (A5) (Parker and Guthrie, 1985).

The mechanistic basis for the loss of specificity observed in the A5 mutant remains to be elucidated. Nonetheless, we have concluded previously that the aberrant cleavage upstream of the intact junction (/GT) is dependent on prior recognition of the 5' consensus sequence, presumably by the yeast equivalent of the U1 snRNP. This argues for the critical role of *trans*-acting factors in determining the sites of subsequent catalytic events. We can now extend this principle to the case of branch site selection. We have shown that when present in an otherwise wild-type actin intron background, the A256 mutation activates branch formation at the upstream, sequence TACTAAG box. This cryptic site, while it is normally not used, has been demonstrated by us (Cellini *et al* ., 1986) to be activated when the wild-type TACTAAC is deleted. This suggests that the A256 mutation disrupts an important recognition contact, and thus allows an otherwise inactive TACTAAC box to compete for binding by a factor which, in analogy to results from HeLa (Chabot *et al* ., 1985; Krainer and Maniatis, 1985), is likely to be the yeast analogue of the U2 snRNP. As a test of this hypothesis, it would be of interest to determine whether A257 (which is currently available only in the Δ6 background) would also lead to activation of teh cryptic TACTAAC box. Since this mutant is phenotypically much tighter than A256, we would predict that (i) its binding site is comparably weaker and (ii) it should thus promote more effective competition by the cryptic element.

The picture that emerges from these considerations is one in which the primary determinants of the efficiency and specificity of the splicing reactions *per se* (Figure 9, Steps 3 and 4) are the strength of the interactions between the splicing signals and *trans*-acting recognition factors (Steps 1 and 2). Indeed, the most important interactions do not necessarily involve the nucleotides at the sites of bond breakage/formation. According to this view, the predominant consequence of mutations which inhibit splicing should be manifested by the decreased binding of these factors, that should minimally include the snRNPs themselves (Black *et al* ., 1985; Chabot *et al* ., 1985; Krainer and Maniatis, 1985). We have previously demonstrated that *Saccharomyces cerevisiae* contains snRNAs (Wise *et al*

., 1983) and, more recently, that a subset of these capped species are 1) essential for splicing *in vitro* (Cheng and Abelson, 1986) and 2) enriched in the spliceosome fraction (Riedel *et al* ., in preparation). An initial test of this hypothesis is to assay the ability of these mutants to assemble a splicing complex *in vitro*.

Spliceosome assembly is rate-limiting in vitro

While only two of the six intron mutants analyzed do not undergo detectable splicing *in vivo* (A1, C1), only a single mutant (C303/305) showed any activity in the standard *in vitro* splicing assay. While the specific explanation for this discrepancy is not known at this time, it is likely that the *in vitro* system is still sub-optimal with respect to one or more reactions in the pathway. As such, it would provide a more stringent test for the ability of a mutant substrate to undergo the respective event. Consistent with this reasoning is the demonstration that C259 undergoes a low-level of splicing *in vitro* only if the extract is first concentrated by ammonium sulfate precipitation, suggesting that the effective concentration of one or more components may be limiting.

To determine whether the failure of these mutants to undergo splicing was due to a block at or prior to the first nucleolytic event in the pathway, 5' cleavage and branch formation (Step 3), the extracts containing each of these precursors were assayed for their ability to form the 40S complex first described by Brody and Abelson (1985). With the exception of C303/305 (see below), no appreciable spliceosome assembly could be detected; rather the mutant precursors sedimented at the 30S position characteristic of wild-type substrates incubated in the absence of ATP. We conclude that each of these mutants is unable to participate efficiently in Steps 1 and/or 2, i.e., signal recognition and complex assembly. It is important to note that this conclusion by no means excludes the possibility that additional steps are also affected *in vivo* . For example, there is ample evidence that the A1 mutant, in addition to accumulating full-length precursor, is also defective in conversion of lariats (Step 4) in the cell. As we have discussed above, the most reasonable interpretation

is that the A1 mutation has pleiotropic effects; the most proximal in the pathway -- and perhaps the one most evident by a kinetic analysis --is the block in complex formation.

In this light it is of considerable interest to contrast the *in vitro* behavior of A1 with that of C303/305, which appears to be selectively inhibited in Step 4 both *in vivo* and in the *in vitro* splicing assay. Not only is this the sole case in which we can detect 40S complex formation *in vitro*, but the efficiency of the C303/305 assembly reaction is indistinguishable from that of wild-type. This suggests that the essential interactions between splicing signals and *trans*-acting factors that govern spliceosome assembly do not require recognition of the 3' terminal G of the 3' junction. These results are consistent with the observations of Rymond and Rosbash (1985) and Reed and Maniatis (1985).

We conclude that requirements for 3' cleavage and exon ligation (Step 4) appear to be highly restricted: the presence of a G at the 5' position of the branch point is essential, while a G at the 3' splice site itself is highly preferred but not absolutely demanded. Moreover, since the 5' branch position also plays a critical role in other reactions, C303/C305 is the only mutation analyzed that specifically affects Step 4 of the pathway.

Implications

The observation that most mutation examined had a marked effect on precursor accumulation *in vivo* and completely inhibited spliceosome assembly *in vitro* argues, as we have discussed above, that the splicing reaction in yeast is dominated by the strength of the interactions between the splicing signals at the 5' splice junction and the branchpoint and one or more trans-acting factors. These interactions are likely to mediate two steps in the splicing pathway, initial recognition (Step 1) and spliceosome assembly (Step 2).

Further experiments are required to distinguish experimentally between these two steps. In the meantime, we would speculate that each of these reactions can contribute to the effects we have observed. The primary sequence and local structure of a splicing signal almost certainly influences Step 1; the behavior of the A256 mutation, for example, is most

plausibly accounted for by a competition governed by these features. C1 and A257 may represent more extreme cases, in which no sufficiently stable trans interactions can be maintained. In addition, we think it likely that the efficiency with which a given intron is spliced will be determined by snRNP-snRNP interactions that may promote, or inhibit, the formation (and/or stability) of the complex (Step 2). In this regard it is perhaps relevant to compare the quantitative effects of identical mutations that have been observed in different yeast introns. The A1 mutation in the *CYH2* intron (Newman *et al* ., 1985) has a much less drastic effect on complex formation and 5' cleavage than the same mutation in actin; conversely, the C259 mutation has a tighter phenotype in *CYH2* than in actin. Conceivably, then, the relative strengths of 5' and branchpoint signals are coordinated, via interactions in *trans*, to set the effective efficiency of any given intron.

The emergent picture is thus one in which a specific three-dimensional architecture is imparted to the intron by a stepwise association of 1) snRNAs with their *cis* signals, and 2) snRNAs and snRNPs with one another, via a combination of RNA/protein interactions. Together with the fact that the nucleolytic events of splicing occur by two sets of reactions in which the total number of bonds is conserved, these considerations suggest the provocative possibility that the mechanism of splicing of nuclear pre-mRNA introns may be fundamentally similar to that in Class I and Class II introns. As recently suggested by Cech (1986), splicing in both systems would occur by successive *trans* esterification reactions. In the latter case, the autocatalytic capacity is inherent in the intramolecular interactions within the intron; in the former, this structure is imparted to the intron by the intermolecular interactions between the signals and the snRNPs. The recent demonstration that Class II introns self-splice via a lariat intermediate makes this suggestion quite credible (Peebles *et al* ., 1986).

A prediction of this model is that the components of the splicing machinery will not include "conventional" enzymes such as the ligase and endonuclease that mediate tRNA

splicing (Peebles *et al.*, 1983; Greer *et al.*, 1983). The genetic and biochemical dissection of this machinery is currently underway.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England Biolabs. 32P-ATP was purchased from ICN; α-P^{32}UTP and α-P^{32} CTP were purchased from Amersham. Reverse Transcriptase was obtained from Life Science. Oligonucleotides (Table 2) were synthesized using an Applied Biosystems machine. RNasin and SP6 RNA polymerase were obtained from Promega Biotech. Yeast media was prepared as described by Sherman *et al* . (1979).

Oligomutagenesis

The template for oligomutagenesis, MP9.sal was construted by insertion of the 1.4 kb SalI fragment from pYAH-I2 (Parker and Guthrie, 1985) into the *Sal*I site of MP9. Oligomutagenesis was preformed by a procedure described in Newman *et al* ., (1983) with some modifications. Oligonucleotide primed extension was followed by transformation of *E. coli* JM101 (Δlac pro supE thi, F:traD36, proAB, lacI^q Z ΔM15) with an aliquot of the extension products without the separation of the closed circular product from the reaction mixture. Screening for the mutant plaques was done as described by Newman *et al* ., (1985). The sequence of each mutation was confirmed by sequencing using oligonucleotide primers that hybridized to regions of the actin gene downstream of the position being mutagenized, by the method of Sanger *et al* ., (1977).

Plasmid and strain construction

Mutations were subcloned into the test vectors by the following procedures. For pYAH-I2, a plasmid was constructed deleting the 1.4 kb *Sal* I fragment. This plasmid, pYAH-I2 (Δ*Sal* I), was cleaved with *Sal* I and the 1.4 kb *Sal* I fragment from the MP9 RF and was

inserted in the correct orientation. This plasmid was introduced into the yeast strain FC8-24D (Parker and Guthrie, 1985) and targeted to the *HIS*4 locus by cleavage with *Xba* I. Integrations were confirmed by Southern (1975) analysis. For pYAHB2, the *Bam* HI - *Sal* I fragment containing the intron was subcloned into the *Bam* HI-*Sal* I sites adjoining a defective *lac*Z gene, CENIII, TRP-ARS1 containing vector pcGL (unpublished) and introduced into the yeast strain NNY1 (Cellini *et al* ., 1986) by transformation. Transformations were done by the method of Ito *et al* ., (1983). For the SP6 constructions, an the *Alu*I fragment containing the intron and portions of exon 1 and exon 2 were subcloned into the *Sma*I site of the Sp6 transcription vector SP65.

Preparation of RNA and primer extension analysis

RNA was prepared as described by Domdey *et al* ., (1984). Primer extensions were performed as described by Domdey *et al* ., (1984). 5 micrograms of poly(A)$^+$RNA was used.

In vitro transcription

Actin transcripts for the *in vitro* splicing reactions were synthesized in a 10 µl reaction containing 20 mM NaCl, 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, and 0.5 mM of each of the unlabelled nucleotides CTP, ATP, GTP and 25 mM UTP and α-P^{32} UTP at 1 mCi/ml, 1000 U/ml RNasin (Promega Biotech), 50-100 ug/ml linearized template (linearized with *Hin*dIII) 400 U/ml Sp6 RNA polymerase (Promega Biotech). To prepare α- P^{32} CTP labeled actin transcripts 20 µl reactions were done containing 5 mCi/ml α-P^{32} CTP with the concentration of the other unlabelled nucleotides being at 0.5 mM. These reactions were done at 37^{o}C for two hours. The full-length transcripts were purified by gel electrophoresis.

In vitro splicing reactions

Standard splicing reactions were carried out as described in Newman *et al* ., (1985). Extract used in this study were either whole cell extracts of the yeast strain EJ101 (Lin *et al* ., 1985) or more concentrated ammonium sulfate fractions of the whole cell extract that had complete splicing activity. Two different fractions were used. One a 35% ammonium sulphate pellet (McFarland and Abelson, unpublished results) for which 2.0 M potassium phosphate was added to a final concentration of 0.25 M to the whole cell extract. Ammonium sulphate was then added to 35% saturation and stirred on ice for 30 minutes. The pellet was recovered by centrifugation at 17,000 rpm for 30 min (Sorvall SS34) and then resuspended in one-tenth the volume of the crude extract used in the fractionation. Dialysis was done against Buffer D (Newman *et al* ., 1985) for four hours with one change of buffer after two hours. The extract was spun in an Eppendorf Microfuge for one minute to remove insoluble material and the supernatant frozen at -70^{o}C. The extracts had complete splicing activity. The other fraction used was a 40% ammonium sulphate cut of the whole cell extract without adjustment of phosphate (Cheng and Abelson, 1986).

RNA structure analysis

The RNase digestions were done according to Domdey *et al* ., (1984). For fingerprinting and secondary analysis, the T1 digest was separated in the first dimension cellulose acetate membrane electrophoresis, pH 3.5, and in the second dimension by homochromatography on polyethleneimine (PEI) cellulose TLC plates. For secondary analysis (Domdey *et al* ., 1984) of the oligonucleotide, elution was done with 30% triethylamine bicarbonate, pH 10, followed by appropriate RNA digestions. Two-dimensional TLC on PE1 plates was done for the T1 and A ribonuclease double digest.

Gradient analysis of in vitro splicing reactions

In vitro splicing reactions with 2-3 x 10^4 cpm (counted as Cerenkov) of precursor were used in a 100 ul splicing reaction containing 40 ul of the 40% ammonium sulphate pellet of

the yeast whole cell extract. Reactions were carried out for 15 min at 23°C and then layered on a 17% to 32% glycerol gradient, containing 20 mM HEPES pH 7.3, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM DTT. Centrifugation of the gradients was done at 39K rpm for five and half hours in an SW41 rotor at 2°C. Fractions were monitored by Cerenkov counting.

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TABLE 1: BIOLOGICAL EFFECTS OF INTRON MUTATIONS

Mutation	Relative β-galactosidase activity	Growth on histidinol
Wild-type	100%	+
Δ6	110%	+
C0(Δ6)	97%	+
C1(Δ6)	0.5%	-
A1(Δ6)	0.4%	-
A5	45%	+/-
T256	98%	+
A256	47%	+/-
A256(Δ6)	8%	nt
A257(Δ6)	2%	-
C259(Δ6)	7%	-
C303/ C305(Δ6)	2.4%	-

Relative values for β-galactosidase activity were derived from four to seven independent measurements for each of the constructs. Assays were performed as described previously (Larson *et al* ., 1983). Mutations in the wild-type intron were compared to the wild-type construct; mutations in the Δ6 intron were compared to the Δ6 control.

TABLE 2: SYNTHETIC OLIGONUCLEOTIDES USED FOR SITE-DIRECTED MUTAGENESIS

Oligonucleotide	Chain length	Sequence
A1	19	5' CTAGAACATATCAGAATCC
C1	17	5' AGAACATAGCAGAATCC
C0	18	5' TAGAACATACGAGAATCC
A256	19	5' CGATGTTATTACATGAGAC
A257	18	5' TCGATGTTTGTACATGAG
C259	16	5' TCGATGGTAGTACATG
C303/C305	22	5' GCAGCAACGTGTAAACATATAA

FIGURE LEGENDS

Fig 1. Intron Mutations and Plasmid Constructions

The starting plasmid, pM9. Δ6 is shown along with an expanded version of the actin intron. Using this plasmid, oligonucleotide site-directed mutagenesis was performed to construct the mutations shown. Mutations are designated by the nucleotide change and the numerical position within the intron (i.e., A1 represents the introduction of an A residue at the first position of the intron). All mutations are present in the 6 background (see Materials and Methods) except for A5, T256 and A256, which are present in an otherwise wild-type intron. Symbols for fusion sequences: open rectangle, actin exons; thin line, actin intron; hatched areas, *HIS*4 gene; dotted areas, E.coli *lac*Z gene. The SP6 promoter is shown schematically as a dark black arrow.

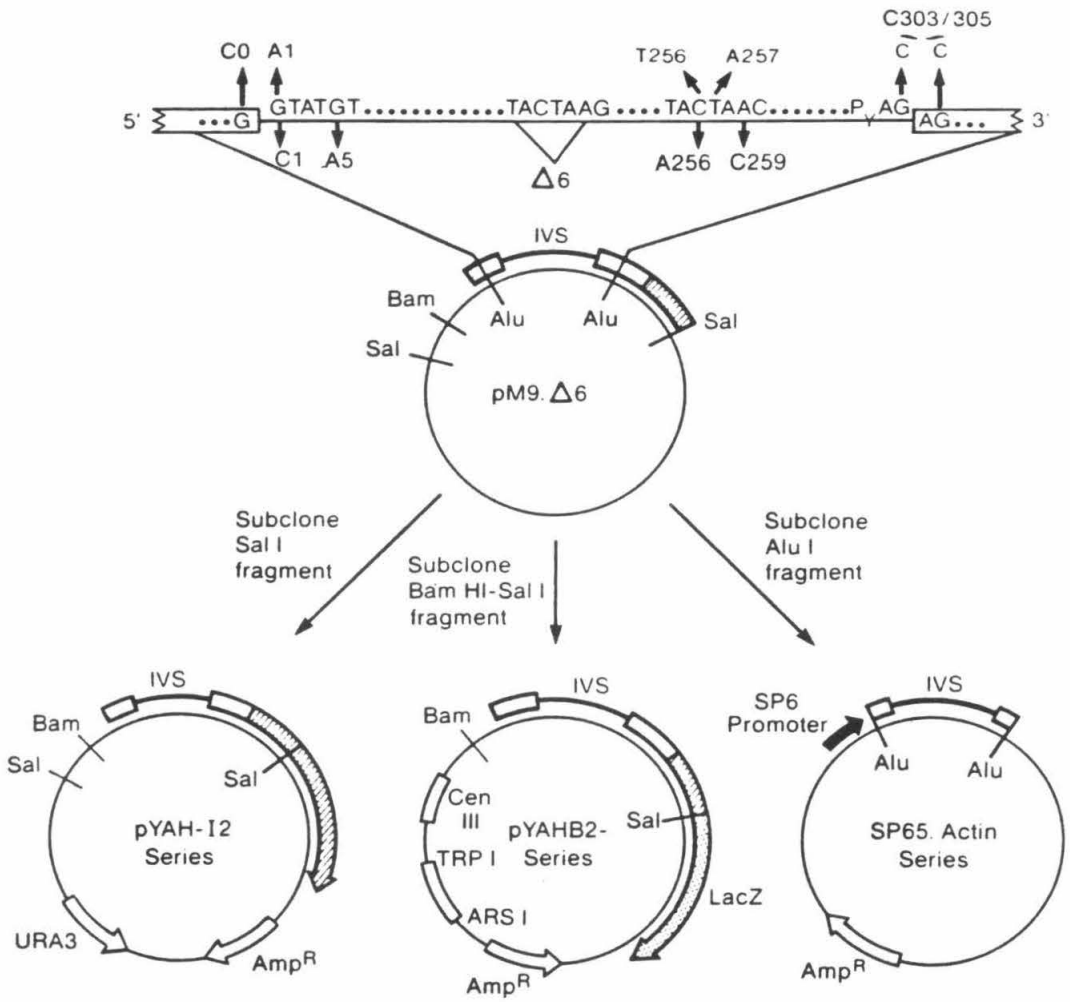

C303/305
C0 A1
T256 A257
C C
5'
...G
GTATGT.....TACTAAG....TACTAAC.....PyAG
AG...
3'
C1 A5
Δ6
A256 C259
IVS
Bam
Alu
Alu
Sal
Sal
pM9.Δ6
Subclone
Sal I
fragment
Subclone
Bam HI-Sal I
fragment
Subclone
Alu I
fragment
IVS
Bam
Sal
Sal
pYAH-I2
Series
URA3
AmpR
IVS
Bam
Cen
III
pYAHB2-
Series
Sal
TRP I
ARS I
LacZ
AmpR
IVS
SP6
Promoter
Alu
Alu
SP65. Actin
Series
AmpR

Fig 2. Primer Extension from the 3' Exon

Poly(A^+) RNA was prepared from FC8-24D transformed with either the wild-type or mutant derivatives of pYAH-I2 (see Figure 1) and was treated to primer extension as described in Materials and Methods. The primer for this experiment is the 21mer, 5' CTCTTCATTACTCAGGCTCGA 3', which hybridizes specifically to the actin-*HIS*4 junction. In the schematic beside the gel, actin sequences are shown as open boxes, intron sequences as a solid line, and *HIS*4 sequences as slashed boxes. Samples in each lane are as indicated by the heading above the gel. Abbreviations are; UT, untransformed FC8-24D, WT, wild-type intron. Size markers are 5' end-labeled *Hpa*II cut pBR325.

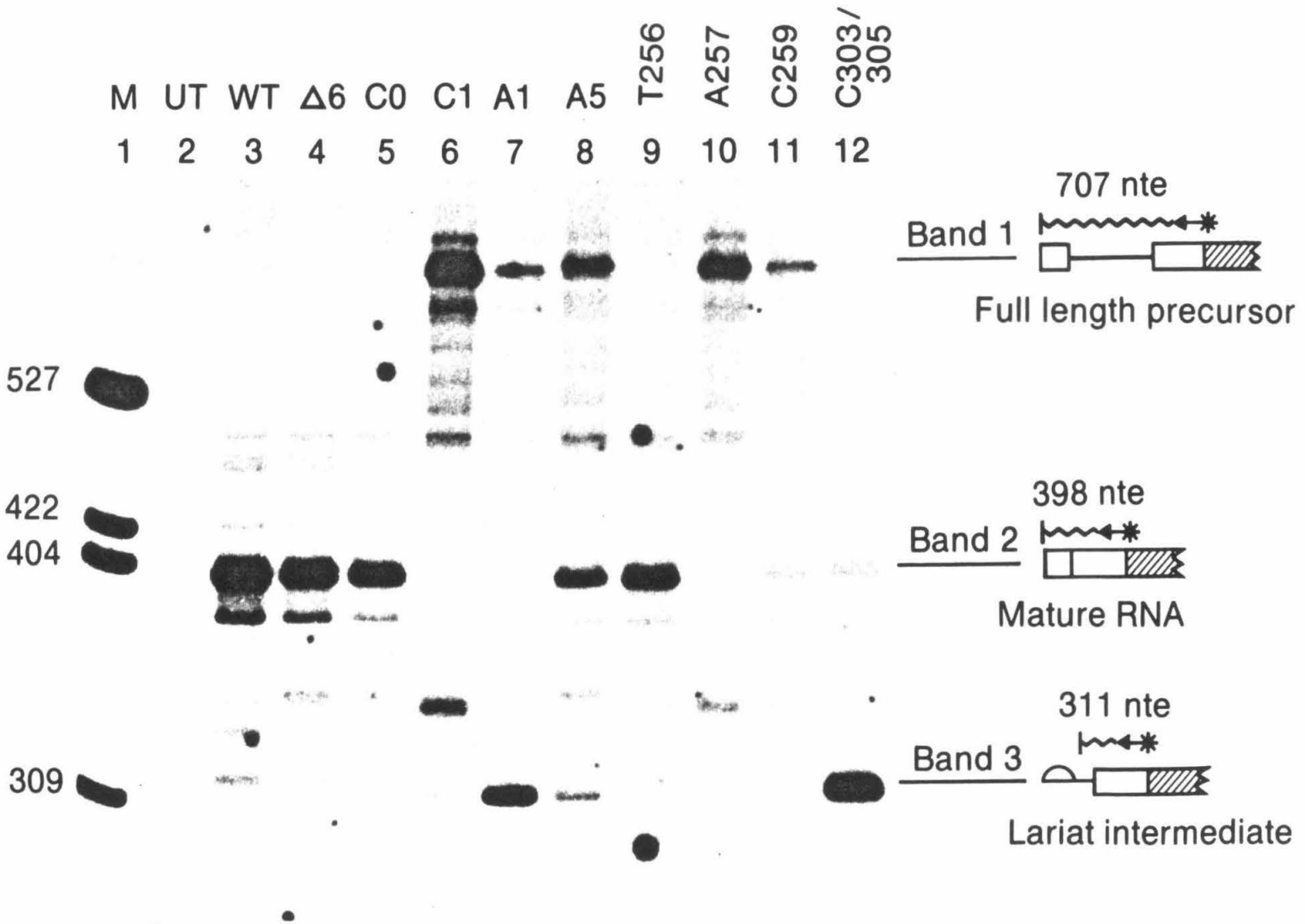

M UT WT Δ6 C0 C1 A1 A5 T256 A257 C259 C303/305
1 2 3 4 5 6 7 8 9 10 11 12
527
422
404
309
707 nte
Band 1
Full length precursor
398 nte
Band 2
Mature RNA
311 nte
Band 3
Lariat intermediate

Fig 3. Primer Extension from a Primer 5' of the Branch Point

Poly(A)$^+$ RNA was prepared from FC8-24D and subjected to primer extension analysis as described in the Materials and Methods. The primer for this experiment is the oligonucleotide, 5' AATTCTTCTTACAGTTAAATGG 3', which hybridizes to the intron 46 nucleotides 3' of the 5' splice site (see schematic). Samples in each lane are denoted above the gel. Because the host strain for these experiments is deleted for the chromosomal actin intron (Parker and Guthrie, 1985) all cDNA products are derived from the fusion transcripts. The sequencing ladder used for positioning primer extension stops was generated by sequencing off M13. Δ6 with the same kinased primer. Lanes are labeled as the nucleotides found in the RNA strand. Size markers are 5' end-labeled *Hpa*II cut pBR325.

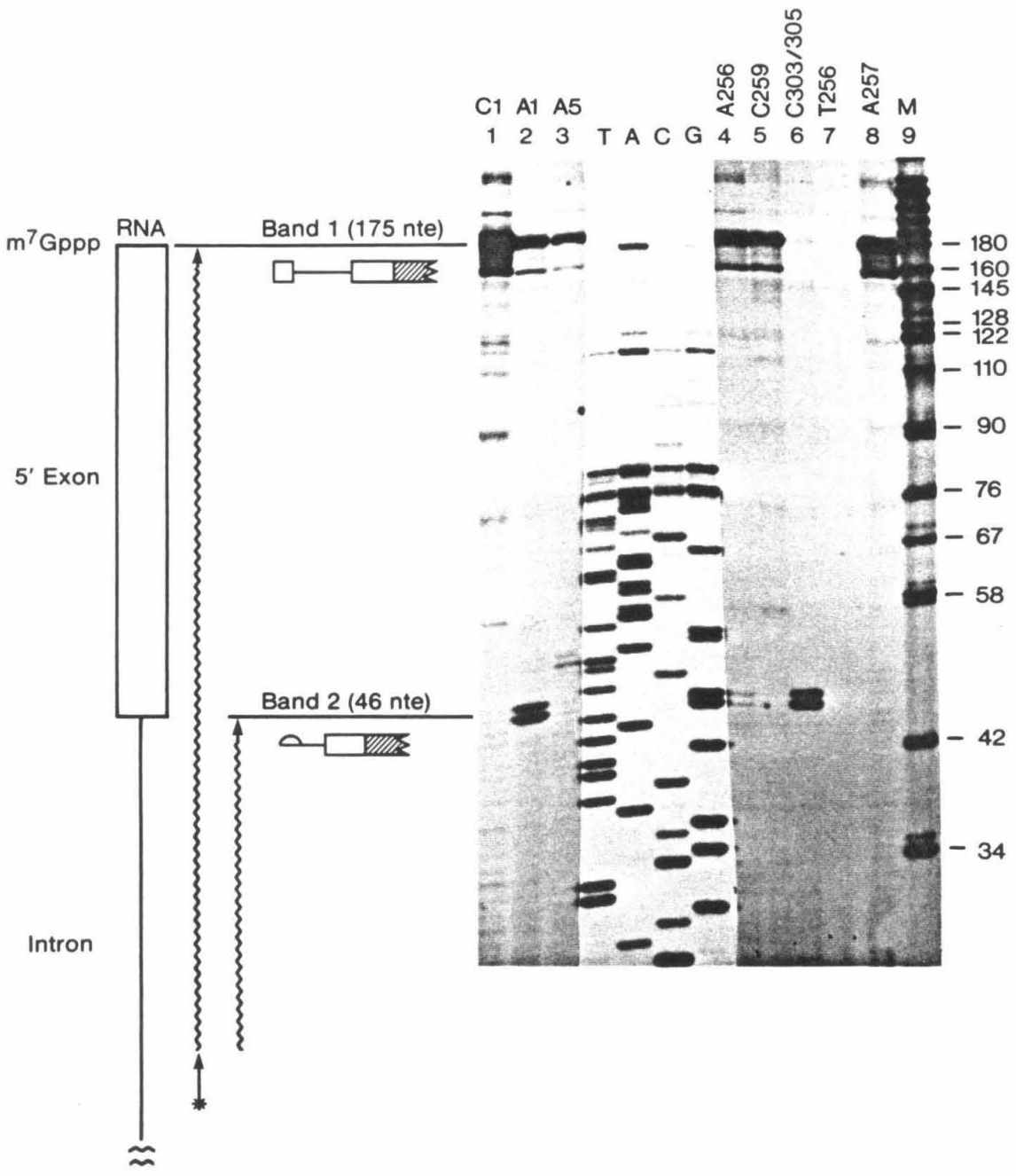
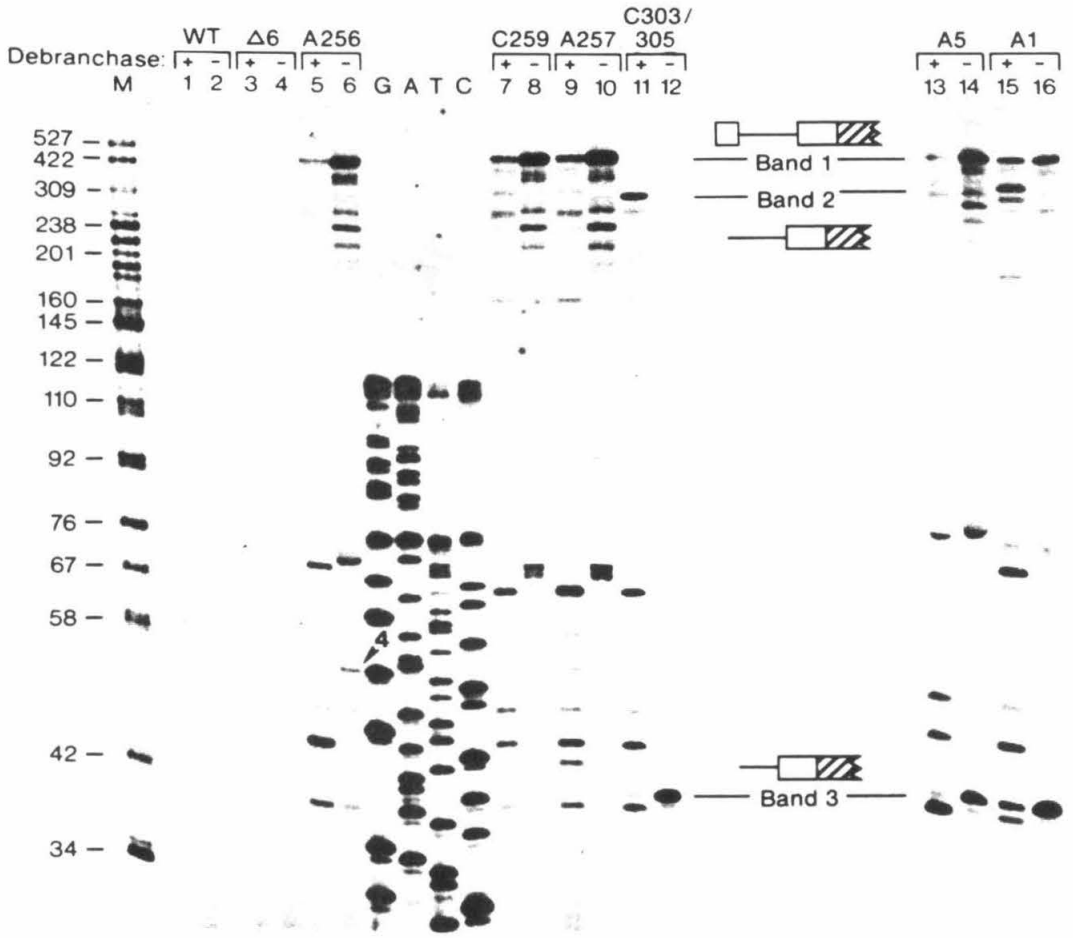

C1 A1 A5
1 2 3
T A C G
A256 C259 C303/305 T256 A257 M
4 5 6 7 8 9
m7Gppp
RNA
Band 1 (175 nte)
5' Exon
Band 2 (46 nte)
Intron
180
160
145
128
122
110
90
76
67
58
42
34

Fig 4. Position of Branchpoints in Splicing Mutations

Poly(A)$^+$ RNA was prepared from the strain FC8-24D transformed with either the wild-type or mutant derivatives of pYAH-I2 (see Figure 1), and was used for primer extension with or without prior debranching as described in Experimental Procedures. The primer for this experiment is the 24mer, 5' CTAAACATATAATATAGACACAAA 3', which hybridizes to the 3' end of the intron. The sequencing ladder used for positioning primer extension stops was generated by sequencing off M13. 6 with the same kinased primer. Lanes are labeled as the nucleotides found in the RNA strand. Size markers are 5' end-labeled *Hpa*II cut pBR325. The cDNA products of approximately 70 nucleotides found in RNA preparation containing full-length precursor correspond to a nine nucleotide helix that appears to generate both reverse transcriptase stalling and specific degradation products in the debranchase reaction.



Extension Products :

No Debranchase

1) Full length precursor (Band 1)

429 nte

2) Lariat intermediate (Band 3)

43 nte

Debranched

1) Full length precursor (Band 1)

429 nte

2) 5' Junction (Band 2)

303 nte

Additional branch in A256 (Band 4)

Normal branch

... TGTTACTAAGTCTCATGTACTAACATCGA ..... TAG

A256

Fig 5. *In vitro* Splicing of Mutant Substrates

The figure illustrates the products of *in vitro* splicing (see Materials and Methods) for either wild-type or mutant substrates. The mutation in the input precursor is indicated above each set of lanes, wt represents wild-type. Bands are as follows: band 1, input full-length precursor; band 2, lariat intermediate; band 3, free exon 1; band 4, excised lariat intron; band 5, mature ligated mRNA. The mature mRNA for the wild-type construct is slightly shorter than the Δ6 construct due to a smaller exon 1. This is a result of being in a different SP6 transcription vector. Similarly, the input precursor for A256 is slightly shorter because it is in the SP64 vector.

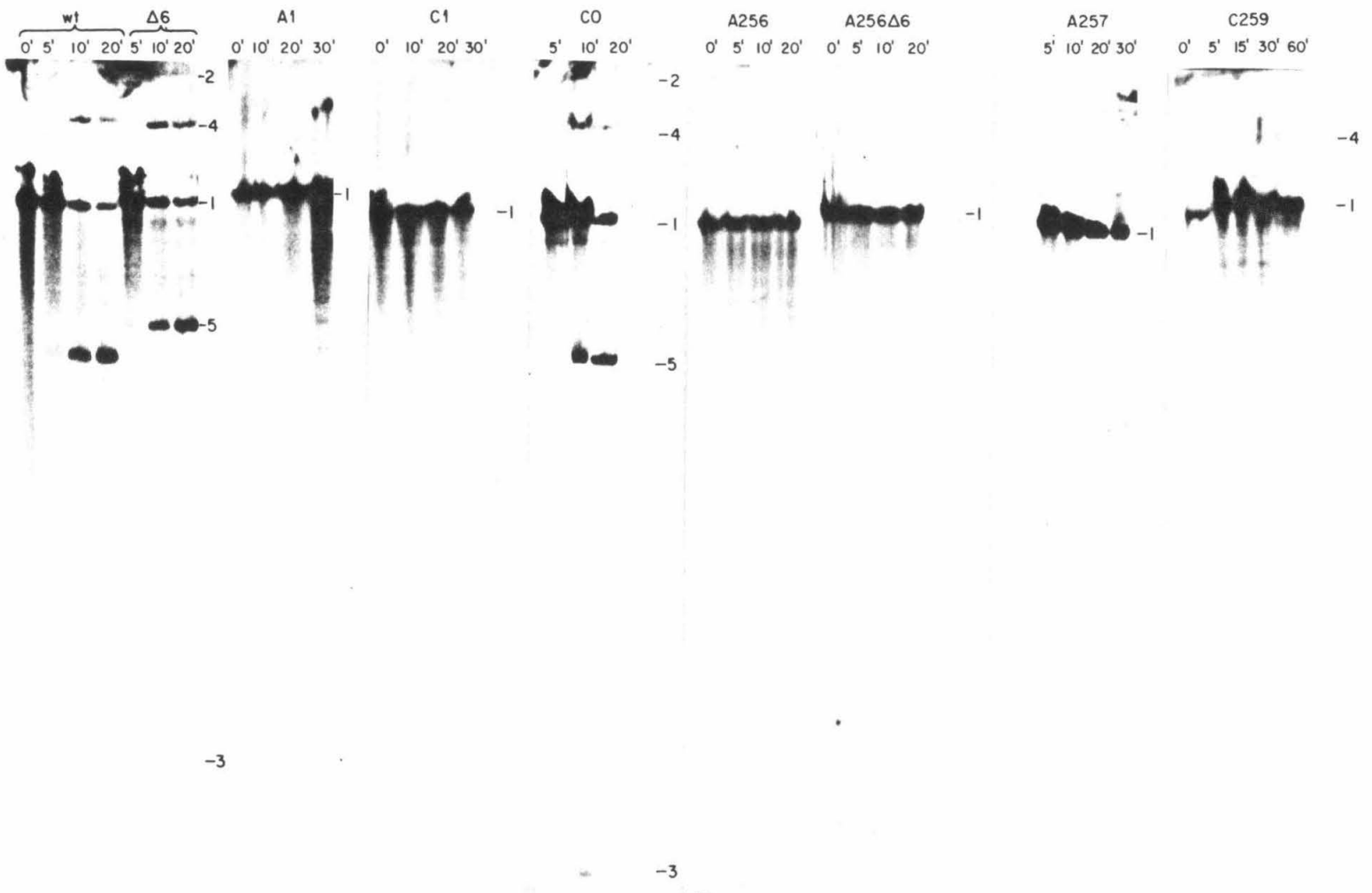

wt
Δ6
A1
C1
CO
A256
A256Δ6
A257
C259
0' 5' 10' 20' 5' 10' 20'
0' 10' 20' 30'
0' 10' 20' 30'
5' 10' 20'
0' 5' 10' 20'
0' 5' 10' 20'
5' 10' 20' 30'
0' 5' 15' 30' 60'
-2
-4
-1
-5
-3

Fig 6. *In vitro* Splicing of the C303/C305 Mutation

This figure illustrates the products of *in vitro* splicing for the C303/C305 mutation. Panel A is using a normal whole-cell extract. Panel B is using a 35% ammonium sulfate pellet (described in Materials and Methods), which allows the detection of some excised intron (band 4). Bands are labeled as follows: band 1, input precursor; band 2, lariat intermediate; band 3, free exon1; band 4, excised intron.

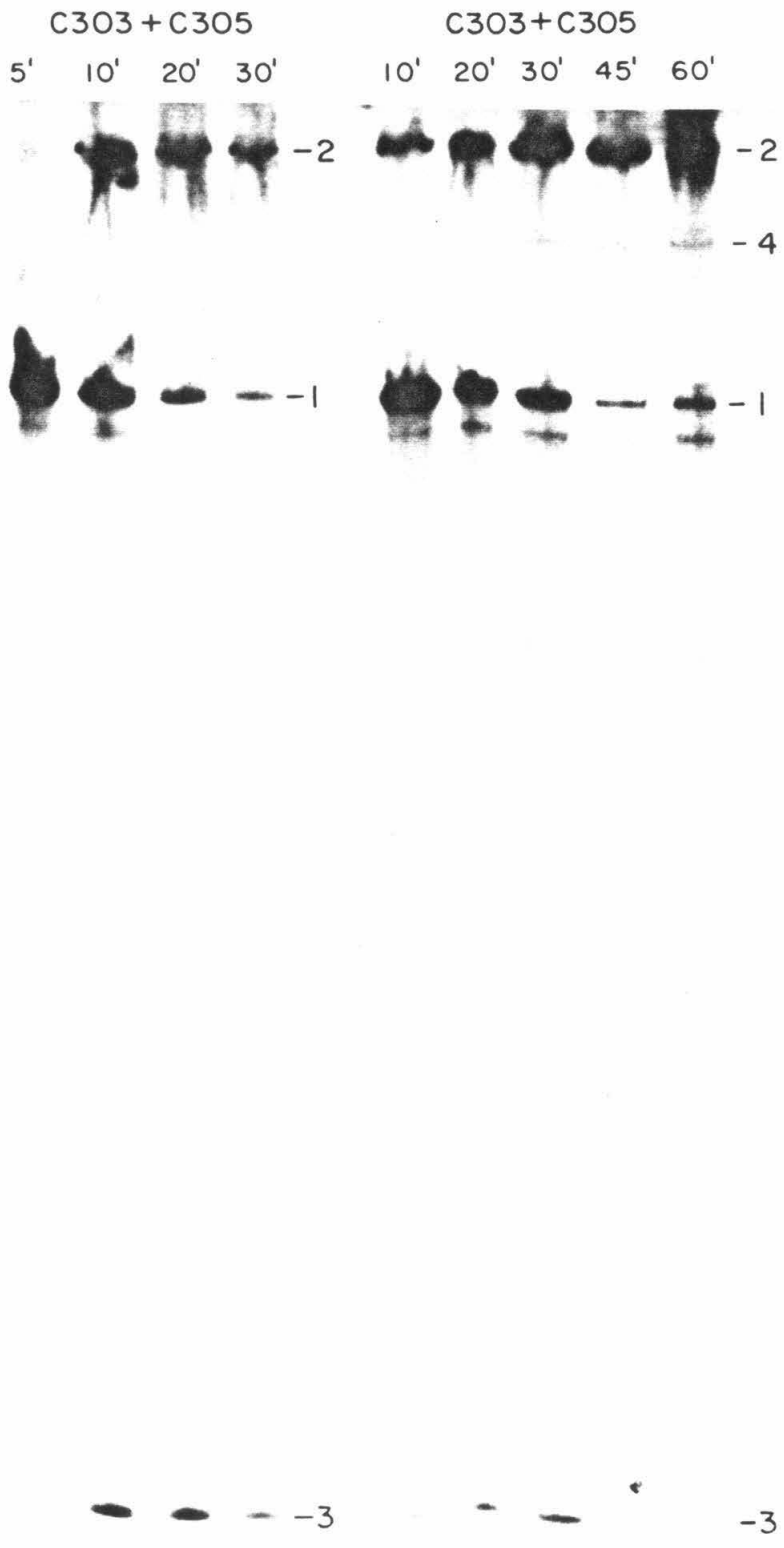

C303 + C305
5' 10' 20' 30'
-2
-1
C303+C305
10' 20' 30' 45' 60'
-2
-4
-1
-3
-3

Fig 7. Fingerprint Analysis of the Excised Intron for C303/C305

The excised intron for Δ6 and C303/C305 mutant (band 4 in Figure 6) was purified and subjected to fingerprint analysis as described in the Materials and Methods. The T1 oligonucleotides derived from the 3' end of the IVS are indicated by arrows.

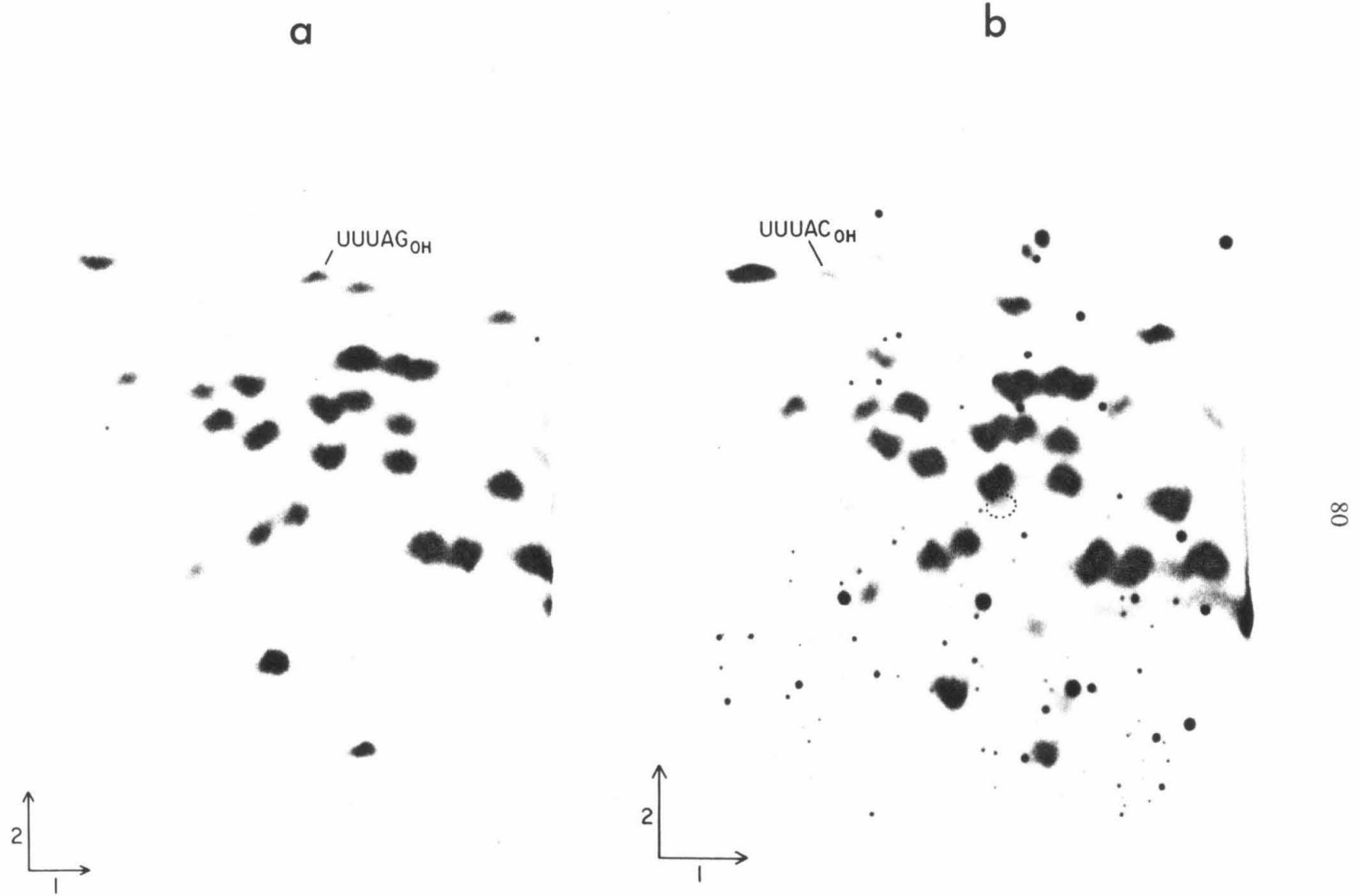

a
UUUAG$_{OH}$
2
1
b
UUUAC$_{OH}$
2
1

Fig 8. Complex Assembly Assays for Mutant Substrates

The figure shows the glycerol gradient profile of hot precursor following incubation in a splicing extract (see Materials and Methods). In panel A the solid line represents the profile in the abscence of ATP, the dotted line represents the profile in the presence of ATP. In all other panels the solid line represents the mutant precursor, the dotted line represents a wild-type control. The input precursor for panal A is the Δ6 construction.

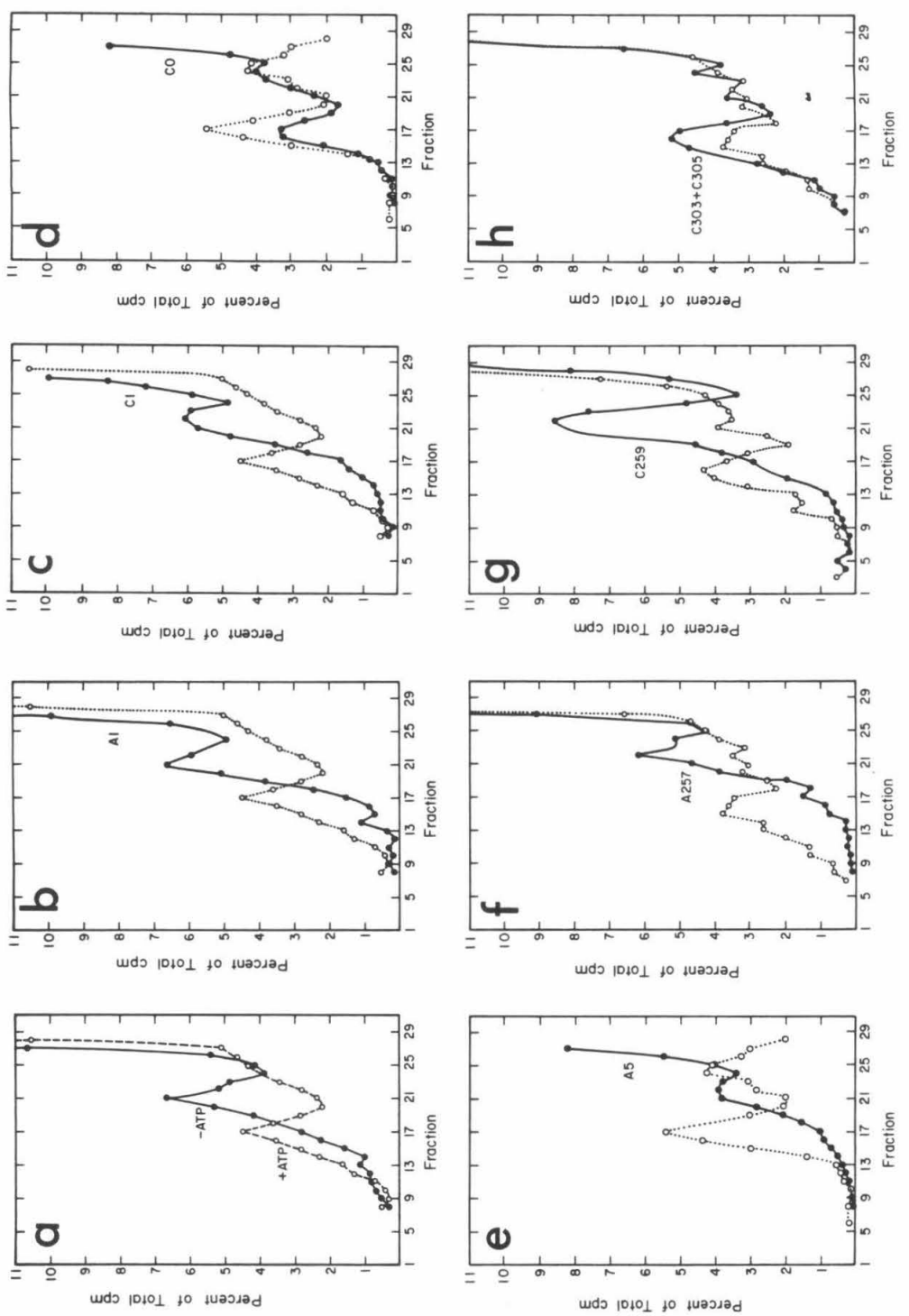
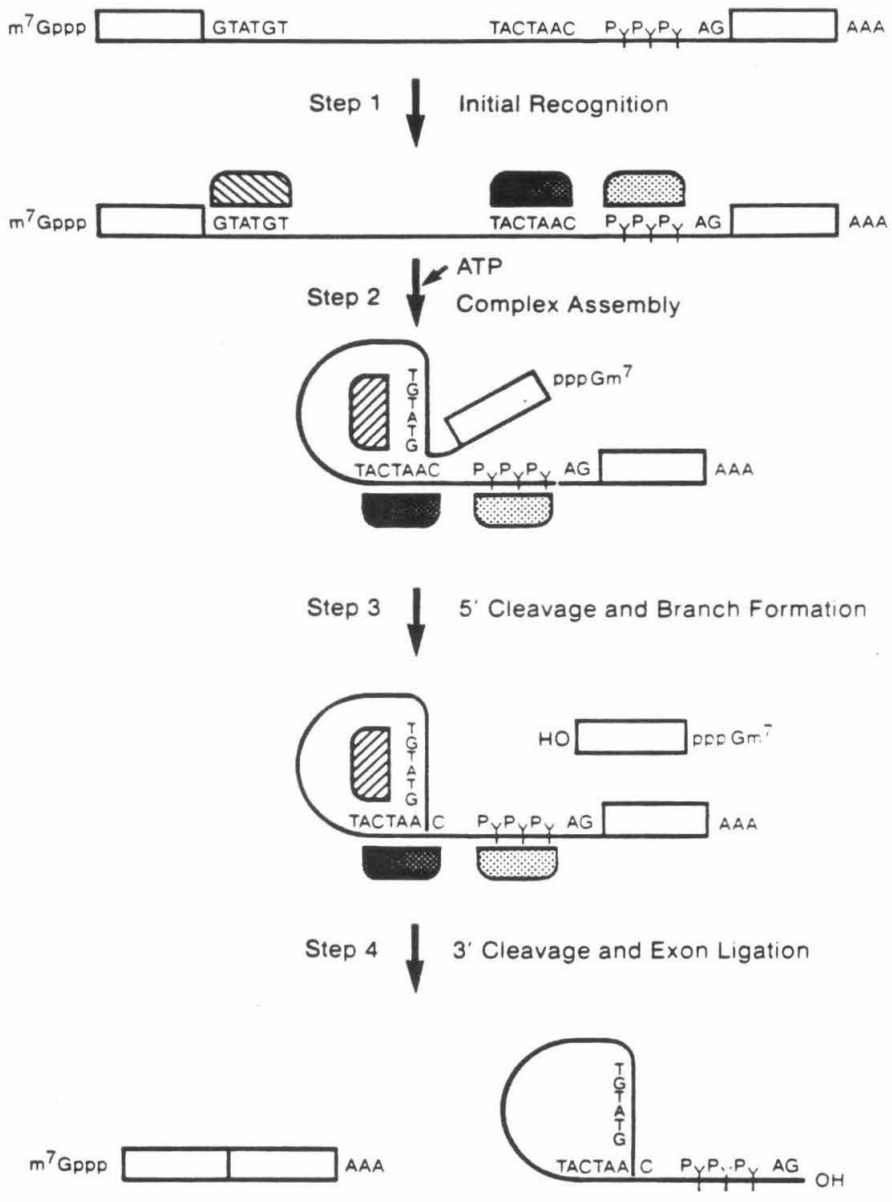

a
b
c
d
e
f
g
h
-ATP
+ATP
A1
C1
C0
A5
A257
C259
C303+C305
Percent of Total cpm
Fraction

Fig 9. Putative Splicing Pathway


m7Gppp
GTATGT
TACTAAC
PyPyPy
AG
AAA
Step 1
Initial Recognition
ATP
Step 2
Complex Assembly
pppGm7
TGTATG
Step 3
5' Cleavage and Branch Formation
HO
TACTAAC
Step 4
3' Cleavage and Exon Ligation
OH

CHAPTER 2

Isolation and Characterization of pre-mRNA splicing mutants of *Saccharomyces cerevisiae*

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ABSTRACT

In this study we report the isolation of temperature sensitive mutants that affect pre-mRNA splicing. A bank of about one thousand temperature sensitive (*ts*) *S. cerevisiae* strains was generated and screened on RNA gel blots. We have isolated eleven new complementation groups [*prp* (*rna*)*17-27*], representing four phenotypic classes of mutants. The majority of the complementation groups share a phenotype of pre-mRNA accumulation, a phenotype also seen in all of the *prp* (*rna*)*2-11* mutants. Three novel classes of mutants were isolated in this study. One class, consisting of two complementation groups, exhibits an accumulation of the lariat intermediate of splicing, with no change in the levels of pre-mRNA. The second class, also represented by two complementation groups, shows an accumulation of the intron released after splicing. The third novel class, comprising one group, accumulates both pre-mRNA and the released intron. All mutants isolated were recessive for the splicing phenotype. Except for two complementation groups all other groups contain alleles where co-segregation of the *ts* defect and the splicing phenotype is observed. This study together with previous isolation of the *prp* (*rna*)*2-11* groups puts at least twenty-one gene products involved directly or indirectly in pre-mRNA splicing.

INTRODUCTION

The precise removal of the intron from nuclear pre-mRNA is an essential process in eukaryotic gene expression. The splice site selection in nuclear pre-mRNA is dictated only by short stretches of sequences at or near the splice junctions (Shapiro and Senapathy 1987). The elucidation of a two-step splicing reaction scheme has come through the development of *in vitro* splicing systems; where the first step is cleavage at the 5' splice site and the formation of a lariat structure, and the second step is cleavage at the 3' splice site and ligation of the exons (for reviews see Green 1986; Padgett *et al.*, 1986 and Sharp 1987). The splicing reaction takes place in a multicomponent structure termed the spliceosome, which

has a sedimentation coefficient of approximately 40-60S (Brody and Abelson 1985; Frendewey and Keller 1985 and Grabowski *et al.*, 1985). The formation of the spliceosome is necessary for the splicing reaction, and its assembly requires the presence of appropriate splicing signals in the pre-mRNA.

A major aspect of research in pre-mRNA splicing is the identification and determination of the function of the splicing machinery. It is now clear that at least five snRNPs (U1, U2, U4, U5 and U6), are involved in splicing of both yeast and higher eukaryotic pre-mRNAs (Guthrie and Patterson 1988; Maniatis and Reed 1987; Steitz *et al.*, 1987). An ordered pathway for the assembly of the spliceosome has been deduced (Cheng and Abelson 1987; Konarska and Sharp 1986; Konarska and Sharp 1987; Lamond *et al.*, 1988; Pilkielny *et al.*, 1986). U1 binding to the pre-mRNA occurs first, and this is followed by the binding of U2, U4, U5 and U6 snRNAs (Bindereif and Green 1987Ruby and Abelson 1988). The snRNP-mediated steps in splicing range from direct base pairing interactions between the snRNAs and intron sequences (Parker *et al.*, 1987; Seraphin *et al.*, 1988; Zhuang and Weiner 1986); to interactions co-ordinated by protein factors (Ruskin *et al.*, 1988; Tazi *et al.*, 1986). At least one hnRNP protein is associated with mammalian spliceosomes and is also required for *in vitro* splicing (Choi *et al.*, 1986; Sierakowska *et al.*, 1986).

Studies on pre-mRNA splicing in *S. cerevisiae* can take advantage of the extensive genetic and molecular approaches that are available for the identification and characterization of mutations in the splicing pathway (reviewed in Vijayraghavan and Abelson 1988; Warner 1987). An important aspect in the genetic analysis of splicing in yeast has been the availability of a set of temperature sensitive mutations in the genes *RNA2* through *RNA11* . By general consensus among the community of researchers working on RNA processing in yeast and the yeast genetic stock center, these mutants will be called pre-RNA processing (*prp*) mutants. These genetic candidates for the components of the splicing machinery have been used in *in vitro* biochemical studies. These experiments have demonstrated the

direct participation of many of the *PRP2-11* gene products in the pre-mRNA splicing process (Lustig *et al.*, 1986). Splicing activity in extracts prepared from the *prp 2, 3, 4, 5, 7, 8* and *11* strains are heat sensitive *in vitro* . The loss of activity was shown to be due to the inactivation of an exchangeable component in each case by *in vitro* complementation of pairs of inactivated extracts. Except for *PRP2* the *PRP* gene products 3, 4, 5, 7, 8 and 11 are required for the assembly of the spliceosome (Lin *et al.*, 1987). Molecular characterization of some of the cloned *PRP* genes has indicated that they code for proteins (Jackson *et al.*, 1988; Last *et al.*, 1984; Lee *et al.*, 1984; Slotyk *et al.*, 1984). Antibodies directed against *PRP8* fusion proteins inhibit *in vitro* splicing reactions and these antibodies have been used to detect an association of the *PRP8* protein and the yeast U5 snRNA (Lossky *et al.*, 1987). In the case of *PRP11* the ^{35}S-labeled protein was shown to associate with the 40S spliceosome and to also associate with a 30S complex (Chang *et al.*, 1988). The role of the other *PRP* gene products are being investigated in several laboratories.

A combination of genetic and biochemical techniques is therefore proving to be extremely useful in the identification of pre-mRNA splicing factors. About 5% of the original Hartwell bank of temperature sensitive lethals have now been shown to lead to defects in splicing, thereby suggesting that a large number of genes are involved. The need for a number of trans-acting factors in pre-mRNA splicing is also apparent from the complexity of the spliceosome and the pre-mRNA splicing process. To isolate more mutants defective in pre-mRNA splicing we have constructed a bank of *ts* yeast strains by chemical mutagenesis. The mutant bank has been directly screened for pre-mRNA processing defects by a Northern blot assay. Genetic characterization including complementation and segregation analysis has been conducted for all the candidates. The screen of about 1000 *ts* lethals has resulted in the identification of several new complementation groups. Some of these new complementation groups show novel phenotypes, implying a role for these gene products in various steps of the splicing pathway.

RESULTS

Generation of a bank of *ts* lethals in *S. cerevisiae* : We chose the parent strain for mutagenesis on the basis of the presence of suitable markers for genetic manipulations, and our ability to use extracts prepared from the parental strains for *in vitro* splicing assays. This feature is important for the subsequent analysis of mutants in the *in vitro* splicing system. About a thousand *ts* strains were isolated by ethyl- methanesulfonate (EMS) mutagenesis, followed by replica plating on rich medium to screen individual mutagenized yeast colonies for mutants able to grow at 23°C, but unable to grow at 37°C. The wild type parental strain grew at both temperatures. The dose of EMS and time of mutagenesis were selected to give between 10 to 30% survival after mutagenesis. The *ts* lethals represented about 1-2% of the survivors. The mutagenesis was done on a pair of congenic strains of opposite mating types resulting in the isolation of half of the *ts* bank of one mating type and the second half in the other. Most of the mutants are expected to be of independent origin obtained from several batches of mutagenesis.

Screening of mutants: We have employed a direct assay of screening the mutants for the presence of intron- containing transcripts, not normally seen in a wild type cell (Fig. 1A). The *prp2-11* complementation groups proved to be controls for the development of a screen that could reproducibly obtain such pre-mRNA splicing mutants. They were used as standards in developing conditions for the temperature shift, RNA preparations and the detection of intron-containing transcripts. Early to mid log phase cells from individual *ts* strains grown at permissive temperature (23°C) were shifted to non-permissive temperature (37°C) for two hours, and harvested. Analysis of the RNAs, present at non-permissive temperature, was done after a rough estimation of total RNA. Approximately equal amounts of total RNA were fractionated on agarose gels, and were subsequently blotted onto nylon filters. Two features of the effects of *prp2-11* mutations on RNA processing are evident. First the unique presence of intron-containing precursors after temperature

shift and second, a decrease in the processed mRNA levels. The levels of mRNA are decreased to 10% to 50% of the levels in wild type cells (data not shown). We therefore chose on the criterion of accumulation of pre-mRNA as an indicator of RNA processing defects, a feature common to all the *prp2-11* complementation groups.

These filters were then probed for pre-mRNA with ^{32}P labeled actin intron sequences. The actin transcripts were probed for the following reasons. Firstly actin is an abundant mRNA, and secondly, actin pre-mRNA is efficiently processed and no pre-mRNA or intermediates of RNA processing are detectable in wild type cells (this study). This is in contrast to another intron-containing transcript, that from the CYH2 locus, in which even wild type cells show readily detectable amounts of pre-mRNA (data not shown). In addition, in order to obtain a clear result for splicing defects, the blots were probed with a labeled fragment from the actin intron. This probe allows the detection of pre-mRNA, lariat intermediate, and the released intron, all of which are not found in wild type cells (Fig. 1B). The detection of intron-containing RNA species is indicative of a mutant phenotype.

Several positives that accumulate intron containing RNAs to varying levels were found (Fig. 2). Of the 1000 *ts* mutants screened, 36 mutants were candidates for defects in pre-mRNA processing and were chosen for re-screening. All candidate strains from the primary screen were then colony purified and re-tested for *in vivo ts* lethality. Subsequently, total RNAs were prepared from these strains, grown at both permissive and non-permissive temperature, for northern analysis. Most of the newly isolated mutants showed a temperature dependent accumulation of pre-mRNA (Fig. 2A lanes c and d; e and f; i and j). However, in some cases a splicing phenotype is evident even at the permissive temperature (Fig. 2 lanes g and h). In these cases there exists a possibility of having generated two independent mutations, one affecting the RNA processing and another leading to the *ts* lethality. The splicing phenotype in most mutants is consistent with pre-mRNA accumulation, except for distinct phenotypes observed in the following mutants. *ts a 107* appeared

to accumulate both the pre-mRNA and intron for actin transcripts (data not shown). *ts a 319* appeared to have a pleitropic effect on pre-mRNA processing at non-permissive temperature: both pre-mRNA and an intron-containing transcript larger than pre-mRNA accumulated (Fig. 2A, lane d). Mutants containing RNA of mobility corresponding to that of the released intron were readily apparent even at the primary screen; these strains did not show any accumulation of pre-mRNA (Fig. 2A , lanes a and b).

Separation of the lariat intermediate from the pre-mRNA for actin transcripts is not sufficiently reproducible in these gels to distinguish between these two species of RNA (pre-mRNA being 1.7 kb; lariat intermediate being ~1.5 kb). Mutants that accumulate lariat intermediates specifically, could be identified through primer extension analysis of the RNAs (see below). The levels of mRNA after temperature shift are reduced in all but one of these *prp* mutants consistent with the appearance of pre-mRNA (Fig 2B, lanes c, d, e, f, g, h, i and j). There is no change in mRNA levels observed for the mutant exhibiting accumulation of the intron (Fig.2B, lanes a and b).

If the mutations isolated by this screen affect the cellular splicing machinery, intron-containing transcripts other than actin would be expected to accumulate. This was tested by probing northern blots for a second intron-containing transcript -RP59 . As expected an increase in pre-mRNA or the lariat intermediate was observed together with a dramatic decrease in the level of the mature mRNA at non-permissive temperature (data not shown). All of these mutants were specific for their effect on pre-mRNA, as non-intron-containing transcripts from the TCM1, PAB1 and STE2 loci did not accumulate (M.Aebi per. comm.).

Primer extension analysis : In order to map precisely the 5' ends of the accumulated RNAs, primer extension analyses were conducted. These experiments would clearly distinguish between mutants that specifically accumulate the pre-mRNA from those which accumulate the lariat intermediate. An oligonucleotide primer, complementary to the sequences in the RP51A intron 70 bases downstream of the 5' splice site (For RP51A se-

quence see Teem and Rosbash (1983)) was used for primer extension. Total RNA prepared from cells at both permissive and non-permissive temperature were used. In wild type cells a very low level of the extension products corresponding to the pre-mRNA or the lariat intermediate of RP51A was observed (Fig. 3A, lanes a and b). In the *prp* mutants *ts a 47, ts a 107 , ts a 449, ts a 514 ,ts α 87* and *ts α 344* , an increased levels of pre-mRNA is seen upon temperature shift (for example Fig. 3A, lanes g and h). Two primer extension products corresponding to pre-mRNA were obtained, representing RNAs initiated from the two transcription start sites of RP51A (Teem and Rosbash 1983). An increased level of the lariat intermediate is seen at permissive temperature in some of the strains that accumulate pre-mRNA at non-permissive temperature. This could be indicative of low splicing efficiency even at permissive temperature.

In the strains *ts a 452 , ts a 503 , ts α 365* and *ts α 487* , only a single extension product corresponding to the 5' end of the RP51A intron is detected (For example Fig 3A, lanes e and f). These strains had exhibited accumulation of large, intron-containing actin transcript (~1.5kb , Fig. 2A, lanes g and h) on northern blots. To verify that this ~1.5 kb actin RNA represented the lariat intermediate of actin, primer extension experiments were done with a oligonucleotide primer complementary to the actin intron sequences 70 nts downstream of the 5' splice site. A dideoxy sequencing experiment, using the same primer, on an actin M13 clone allowed a precise mapping of the 5' splice site. These experiments confirm the accumulation of the lariat intermediate of the actin transcript (Fig. 3B, lanes d and e). The primer extension experiment with RP51A transcripts and actin transcripts indicated that in these mutants there is no increase in the level of full length precursor. This suggests that, in these mutants, the first step of splicing : cleavage at the 5' splice site proceeds normally, but the second step of splicing is defective. This phenotype is evident at both permissive and non-permissive temperature and does not affect the precision of cleavage at the 5' splice site. These results also confirm that the primary defect in

these strains is accumulation of the lariat intermediate of splicing with no effect on the levels of the pre-mRNA.

The mutants *ts a 278* , *ts a 391* , *ts α 45* and *ts α 397* , accumulate the released intron specifically (Fig. 2A, lanes a and b). Primer extension again demonstrate a 5' terminus in these accumulated RNAs corresponding to the 5' end of the intron. (For example Fig3A, lane c and d; Fig 3B lanes b and c) The accumulation of the intron was observed at both permissive and non-permissive temperature. Reverse transcription with an oligonucleotide that hybridizes 3' to the branch consensus of RP51A or actin indicates that these molecules are branched at the correct site and therefore do not represent imprecisely spliced or branched molecules (data not shown).

Complementation analysis: The mutants picked by this screen were back-crossed to the appropriate congenic wild type strain of the opposite mating type and heterozygous diploids were obtained. The putative diploids were tested for diploidization and for the *ts* phenotype. All diploids were temperature resistant implying that in all these strains the *ts* loci were recessive. No dominant *ts* mutants were isolated in this screen. In order to define which of the newly isolated mutants belonged to known complementation groups, each mutant strain was crossed to *prp2* through *11* mutants of the opposite mating type, and the diploids were isolated on the appropriate selective media. The diploids were subsequently screened at permissive and non-permissive temperature. Non-complementing diploids exhibit a *ts* phenotype and thus show that the pair of recessive mutants belong to the same complementation group (Fig 4). The complementation analysis showed that six mutants were *prp2* , seven were *prp3* , six were *prp6* and two were *prp9* . No new isolates of *prp4, 5, 7, 8* or *11* were obtained (Table 1). Mutants that complement the *prp2-11* (all pairs of diploids are *ts* $^{+}$) were taken to represent new complementation groups (see Table 1). *ts a 100* , a mutant that accumulates pre-mRNA, had a mating defect and hence has not been assigned to any group. We now assign all the new mutants to complementation

groups. First, the MAT*a* and MAT α mutants were crossed. We determined that the mutants *ts a 452* , *ts a 503* and *ts*α *491*belong to the same complementation group. For the remaining mutants, which were in one mating type, corresponding mutants of the opposite mating type were constructed. We then crossed the Mat *a* mutants with Mat α mutants and assigned complementation groups. All of these complementation results are based on the assumption that in these mutants the *ts* defect and the splicing phenotype were being caused by a mutation in the same gene. In our segregation analysis (see following section) two complementation groups were represented by non-*ts* alleles (Table 1). Re-affirmation of their complementation group assignment was made after analysis of the RNA for the splicing phenotype in the appropriate diploids. Table 1 summarizes the results of the complementation analysis. We find eleven new complementation groups. These groups can be divided into four phenotypic classes: 1) six complementation groups that accumulate precursor; 2) two complementation groups that accumulate lariat intermediate; 3) two complementation groups that accumulate the released intron; and 4) one complementation group that accumulated both pre-mRNA and intron .

Segregation analysis: Mutants that complement *prp2-11* were analyzed further to determine if single-site mutations were responsible for splicing phenotype and the temperature sensitivity. The diploids that were heterozygous for a *prp* mutant were sporulated. Tetrad analysis showed that in all cases the *ts* defect segregated 2:2 (ten to twenty tetrads analyzed in each case), as did all other heterozygous markers, indicating a defect in a single nuclear gene. Co-segregation of the splicing phenotype with the *ts* defect was also analyzed for all the new complementation groups. RNA from several tetrads (in each case six to nine tetrads) and from the heterozygous diploid were analyzed after temperature shift on northern gels (Fig. 5 panels A, B, C and D). Appearance of the splicing defect in the *ts* spores is indicative of co-segregation of the two phenotypes. The heterozygous diploids did not display a gene dosage effect for both the *ts* and *prp* phenotype (data not shown). Co-seg-

regation was observed for the precursor accumulating strains *ts a 47* (Fig. 5a), *ts a 107* (Fig. 5b), *ts a 319* , *ts a 514* , *ts α 87* and *ts α 344* . The lariat intermediate accumulating mutants *ts a 503* (Fig. 5c) and *ts α 365* -representing the two complementation groups- also displayed co-segregation of the splicing and *ts* phenotype.

Among the intron-accumulating mutants only one *ts α 397* (Fig. 5d), exhibited co-segregation of the splicing and *ts* phenotypes. The accumulation of intron in the strains *ts a 278, ts a 391* and *ts α 45* was caused by a single nuclear recessive gene but was not linked to the *ts* defect. This result implied that the four isolates of the intron- accumulating mutant possibily represent one complementation group with *ts* and non *ts* alleles. Diploids obtained from crosses of each of the intron mutants to all the others and also to all the splicing mutants were analyzed for the presence of the intron phenotype at non-permissive temperature. Intron accumulation phenotype was observed only in diploids obtained from pairwise crosses using *ts a 278* , *ts a 391* and *ts α 45* . These results indicated that the intron mutants *ts a 278, ts a 391* and *ts α 45* represent one new complementation group and the mutant *ts α 397* , another (Table 1).

The precursor accumulation in the mutants *ts a 449* and *ts α 377* was not linked to the *ts* lethality, and the isolation of these mutants as *ts* was just serendipitous. An analysis of the RNA in diploids similar to that done for the intron mutants was conducted. These results indicated that the mutant *ts a 449* represented a new complementation group and is not a non-*ts* allele of another complementation group. On the other hand *ts α 377* proved to be a non-*ts* allele of *prp2* , a previously known complementation group.

DISCUSSION

The enumeration, identification and determination of function of the splicing componentsis essential for the complete understanding of the pre-mRNA splicing process. The complexity of the spliceosome is revealed by biochemical work indicating the involvement of five

snRNPs (U1, U2, U4, U5 and U6), hnRNPs and several protein factors. The extensive repertoire of genetic techniques applicable to the yeast *Saccharomyces cerevisae* allows the combined approach of biochemistry and genetics in analysis of pre-mRNA splicing in yeast. Analysis of the yeast mutants in 10 complementation groups (*prp2-11*) over several years has made it clear that primary defect in these strains is the accumulation of intron containing pre-mRNAs at non-permissive temperature (Miller 1984; Rosbash *et al.*, 1981; Teem *et al.*, 1983). The direct participation of many of these gene products comes from demonstration *in vitro* that seven of these complementation groups can be specifically heat inactivated. Further work on these mutants in both *in vitro* and molecular characterization of the wild type loci been of tremendous value in understanding splicing.

Our direct screening of a temperature sensitive bank of yeast strains for splicing mutants has resulted in the isolation of four classes of mutants affecting different stages of the splicing reaction pathway. The major class of six new complementation groups share the phenotype of the *prp2* to *11* groups (pre-mRNA accumulation). Two novel classes affecting late events in splicing were obtained. One class accumulates the lariat intermediate of splicing and the other accumulates the released lariat intron, with no significant increase of the pre-mRNA. One mutant was obtained that could be classified as both a pre-mRNA and an intron-accumulating strain. Such a phenotype could result from the lack of recycling of a early-acting splicing component. The association of such a component with the released intron, after splicing, could account for the stabilization of the intron.

New *ts* mutations define genes involved in various stages of pre-mRNA splicing: The *prp2-11* mutants were initially identified as being defective in RNA metabolism and their isolation was not due to a specific screen for mutants in pre-mRNA splicing (reviewed in Vijayraghavan and Abelson 1988; Warner 1987). Of the initial collection of *ts* lethals generated by nitrosoguanidine mutagenesis about 5% of the mutants were defective in splicing (Hartwell *et al.*, 1970). We have screened our *ts* bank specifi-

cally for identification of mutants in pre-mRNA splicing. The screening of the *ts* mutants for the presence of intron-containing transcripts was a rational approach because all of the previously identified complementation groups (*prp2* to *11*), shared the phenotype of pre-mRNA accumulation at non-permissive temperature. We have isolated 36 mutants defective in pre-mRNA splicing that represented both old and new complementation groups.

The majority of the new complementation groups shared the same phenotype of pre-mRNA accumulation as seen in the *prp2-11* mutants. These mutants presumably act early in splicing. One complementation group displayed a pleiotropic effect on mRNA processing. The presence of intron-containing transcripts larger than the pre-mRNA was seen for both actin and RP59. Further analysis of this mutant phenotype would be essential to determine the primary defect in this strain. The isolation of a large number of complementation groups affecting the early steps of the splicing pathway could be due to the requirement for several factors early in spliceosome assembly. These could range from components that are direct participants in the splicing machinery, to components that perform essential modifications of the spliceosomal components, or to components of hnRNP complexes and factors involved in only the assembly of the spliceosome and not in later functions of the spliceosomes. An alternative reason could be the presence of shared components between the early and late steps of the splicing pathway.

The identification of pre-mRNA processing mutants by this approach does not seem to be limited to those that can be isolated as *ts* alleles alone. From our *ts* bank we have isolated one new complementation group that accumulates pre-mRNA, but results in a splicing defect without a severe growth defect at 37°C. The explanations for this phenotype are unclear. In addition we have obtained mutants (discussed below) that accumulate intron, but are not *ts*, and also a non-*ts* allele of a previously identified mutant-*prp2* .

Not all of the *rna 2-11* complementation groups were represented in this screen of EMS induced *ts* mutations. However, there exists a similarity in the distribution of allele numbers in the *prp2* , *prp3* and *prp9* groups between this and the previous study (Harwell

et al., 1970) . In both cases a significantly higher number of *prp2* and *prp3* alleles were obtained than alleles in other complementation groups. One difference is the higher frequency of isolation of *prp6* in this study. *prp7, prp8* and *prp11* were represented by single isolates in the Hartwell screen and no alleles of these mutants were picked up in this screen. This might be reflected in the number of mutants screened (~1000) after our moderate mutagenesis compared to the screen of the heavily mutagenized bank (400).

The direct screen employed has also obtained new complementation groups that affect late events of splicing. Four complementation groups exhibit novel phenotypes of lariat intermediate or intron accumulation . The Hartwell screen employed for the isolation of the *prp2-11* mutants was based on the ratios of protein/RNA incorporation being >4, hence it is not unexpected that no mutants that accumulate intron were obtained ( ). The levels of mRNA, and hence protein synthesis in the intron-accumulating mutants is probably unaffected within two hours of temperature shift. The isolation of these new complementation groups in our search for splicing mutants might be an effect of both the direct screen employed and the mutagen used.

Trans-acting *ts* mutations result in lariat intermediate accumulation : The isolation of mutants with a novel phenotype affecting only the second reaction of splicing allows an opportunity for the investigation of factors required specifically after the spliceosome assembly and the first step of splicing. Two complementation groups with this phenotype have been obtained. All mutants in these groups are temperature sensitive. The precision of the 5' splice site and the branch point selection is not affected and utilization of the wild type 5' splice site and branch formation site is observed. No change in the levels of the pre-mRNA occurs; hence, the effects seem to be specific for the second step of splicing and the presence of the lariat intermediate in splicing is probably not due to slower splicing. The alleles are partly defective at permissive temperature and show increased levels of the lariat intermediate. The second step of splicing leading to formation of mRNA

and lariat intron takes place, although inefficiently , at permissive temperature. A substantial decrease in second splicing reaction and hence the mRNA levels takes place upon temperature shift.

These are the only trans-acting mutants known to act in the splicing pathway after the 5' splice site cleavage has taken place. From the previous studies on the analysis of the spliceosome assembly, one expects an accumulation of the active spliceosome containing the exon1 and the lariat intermediate (Cheng and Abelson 1987; Pikielny *et al.*, 1986). None of the *prp2-11* mutants undergo any of the splicing reactions and most are required early in spliceosome assembly (Lin *et al.*, 1987). Of the *prp2-11* mutants, the *PRP2* gene product is the only one known to be required after the formation of a late intermediate of spliceosome assembly (Lin *et al.*, 1987). Heat inactivation experiments with *prp2* had demonstrated the accumulation of spliceosomes containing the pre-mRNA with both steps of splicing blocked. The newly defined complementation groups that accumulate the lariat intermediate must represent gene products that act after *PRP2* in the spliceosome assembly pathway. Chasing experiments with heat inactivated *prp2* spliceosomes have led to the proposal that two distinct sets of factors are required for the second step of splicing reactions (Lin *et al.*, 1987). One of these factors, fraction III, had been identified earlier through fractionation of wild type extracts (Cheng and Abelson 1986). The second set of factors termed c_n comprise an unidentified set of factors. The *ts* mutations in two complementation groups accumulating the lariat intermediate possibly result from mutations in one of these factors predicted to act late in the splicing pathway.

Stabilization of the released intron: Apart from the isolation of mutants required for the second step of splicing, new complementation groups were obtained with novel phenotype identifying gene products required after splicing has been completed. The phenotype of intron accumulation was observed in two complementation groups. One group is represented by a single allele and is *ts* , while the other consists of three alleles and none of

them are *ts* (Table 1). Wild type cells appear to degrade the intron very rapidly, with no detectable amount of the released intron in the cells. The accumulation of the intron implies that it must be stabilized. The intron-accumulating phenotype does not result from the accumulation of aberrantly formed lariats due to the imprecise utilization of the 5' splice site or the branch acceptor nucleotide (Fig. 3). The stabilization of the intron could result from its association with spliceosomal components due to a defect in the disassembly, or from a defect in the debranching function itself. Analysis of the biochemical phenotype should lead to a better understanding of the nature of these mutations. An additional mutant (*ts 107*)shows accumulation of both pre-mRNA and some intron. It probably results from a mutation in a factor required early in splicing. The accumulation of intron in this mutant may result from the continued association of this factor with released intron. Altenatively, this mutation may somehow affect the recycling of spliceosomal factors after the release of the intron.

Isolation of new alleles of *prp2*, *prp3* , *prp6* and *prp9* : The 21 new alleles corresponding to the previously identified complementation groups (*prp2-11*) were identified solely on the basis of complementation of the *ts* or *prp* phenotype in the diploid. In multicomponent systems, like pre-mRNA splicing, the possibility that the *ts* or the *prp* phenotype in the diploid is due to unlinked, non-complementation needs to be considered (for example Atkinson 1985; Stearns and Botstein 1988). Thus, it is possible that some of the mutants now thought to be in the *prp2-11* complementation groups could actually represent new mutations in components interacting with the *PRP 2-11* gene products. This question can easily be addressed genetically through the recombination analysis of spores from temperature sensitive diploids. The isolation of any non-complementing unlinked mutants would be extremely interesting as these mutants might define components that interact with *PRP2, 3 , 6* and *9* gene products. These mutations resulting in pre-mRNA accumulation, could define components that are themselves a part of the splicing machinery.

The assignment of *ts α 100 , ts α 200* to the *prp9* complementation group is tentative since it is uncertain that the original *prp9-1* mutation was due to a single site ts mutation (Hartwell *et al.*, 1970). Confirmation of the assignment of the putative *prp9* alleles should be easily done once the genetic analysis of the original *prp9-1* allele is complete.

In conclusion, we have isolated more splicing mutants affecting various stages of the splicing pathway. Of these, a few complementation groups (*prp2, prp17, prp18, prp25* and *prp26*) specifically affect splicing after the spliceosome assembly. The isolation of multiple alleles in some of these groups suggests that few components are required specifically for the later stages of splicing. Two avenues of research are now open to further characterize the new mutants. The co-segregation of the *prp* and the *ts* phenotype provides a good oppurtunity to study the function of these gene products through rapid molecular cloning. In parallel, the *in vitro* characterization of the mutants would also be useful in giving a more detailed understanding of these phenotypes. Both of these complementary approaches are easily achieved in yeast with the existence of *in vitro* systems and the extensive molecular genetic techniques.

Footnote : The genetic designation of the *rna2-11* mutants isolated by Hartwell *et al.* 1970 is being changed by a general consensus among the yeast RNA processing community to *prp* for pre-RNA processing. This nomenclature would clarify that these *prp* (*rna*) mutants are different from the small nuclear RNAs that also play an essential role in pre-mRNA splicing. Most of the *prp* (*rna*) that have been analyzed code for protein components of the splicing machinery.

MATERIALS AND METHODS

***Yeast strains*:** Table 2 lists the strains used in this study. The source of these strains is described in Lustig *et al.*, (1986).

***Genetic manipulations*:** The media, rich (YPD), omission, presporulation and sporulation were prepared as described in Sherman *et al.*, (1986). Complementation analysis was carried out by mixing pairs of *rna* mutants of opposite mating type on YPD plates and allowing mating and diploid growth at 23°C overnight. The diploids were obtained from minimal plates supplemented for their selection . The diploids were then tested for growth at 37°C on YPD plates. In cases where necessary, complementation of the *rna* phenotype in the diploids was assessed by northern analysis. Diploids for the analysis of dominant *vs* recessive nature were obtained at permissive temperature from selective media. Sporulation of the heterozygous diploids was done at 23°C and dissection of spores was done by standard procedures also at 23°C.

***Mutagenesis*:** The two strains, wild type for growth at 37°C : SS330 and SS328 were mutagenized with the alkylating agent ethylmethanesulfonate (EMS) essentially as in Moir *et al.*, (1982). Stationary phase cells were resuspended at 10^8 cells/ml in 0.1M sodium phosphate buffer pH7.0 and were treated with 3% EMS for about 60 minutes. This treatment was calibrated to give 10-30% survival for these strains after the procedure. Mutagenized cells were first diluted 40-fold in sterile 5% sodium thiosulfate to inactivate the mutagen. This was followed by dilutions in sterile water and plating for growth on YPD plates at 23°C. Plates with ~200 colonies were replica plated on pre-warmed YPD plates and left overnight at 37°C. Colonies that grew poorly or did not grow at 37°C were retested for the growth defect and the strains that still exhibited poor or no growth at 37°C were included in the temperature sensitive strain collection.

***Temperature shift and preparation of RNA*:** Each of the *ts* mutants was grown in a 2ml. YPD culture to early-to-mid log phase of growth (A_{600} 0.5 to 2.0) and then shifted to non-permissive temperature 37°C for two hours. Cells were pelleted and then frozen in dry ice. As a control for the temperature shift experiment either a *prp2* or a *prp5* strain was

used as a control. Extraction of the total RNA from the cell pellet was done by the method of hot phenol extraction similar to that of Domdey *et al.*, (1984). Cell pellets were resuspended in 50mM NaOAC pH5.3, 10mMEDTA vortexed and SDS was added to 1%. The cell suspension was vortexed and extracted with phenol (equilibrated with 50mM NaOAc 10mM EDTA) at 65°C for five minutes. Following centrifugation the aqueous phase was transferred and extracted with equal volume of phenol:chloroform::1:1, at room temperature for five minutes. After centrifugation the aqueous phase was brought to 0.3M NaOAC and the RNA precipitated with 2.5 to 3 volumes of ethanol. The RNA yield was usually 50-200 micrograms from this small scale prep. Rescreening of the positives from the first screen was done at both temperatures. Here an aliquot of the mid log phase cells was shifted to non-permissive temperature for two hours and the rest of the culture maintained at permissive temperature. Extraction of RNA was done as described above from both cultures.

***Northern analysis*:** Approximately 10 micrograms of total RNA from each mutant at non-permissive temperature was used for northern analysis on formaldehyde-agarose gels. The RNA was added to formamide dye mix (50% formamide, 6%formaldehyde, 50mM HEPES pH7.4, 1 mM EDTA, 20% glycerol and 0.1% of the dyes bromophenol blue and xylene cyanol) and heated at 65C for two minutes before loading on gels. The electrophoresis was done in 1.2% agarose gels in 50mM HEPES 1mM EDTA 6% formaldehyde. Following electrophoresis the RNA was blotted on to Gene-Screen membranes in 25mM sodium phosphate pH6.5. The RNA was cross-linked on the membranes as described in Cheng and Abelson (1987).

Prehybridization, hybridization and washing of the filters was done as described in Cheng and Abelson (1987). The blots were probed with a ~200 bp fragment of the actin intron (XhoI to ClaI of actin intron), labeled by the random primer extension method of Feinberg and Vogelstein (1983) and used for the detection of the pre-mRNA, lariat inter-

mediate and the intron. The detection of the mRNA and the pre-mRNA was done with the labeled fragment of the actin clone. Reprobing of the blots was done after removal of the probe by boiling in 0.1x SSC, 0.1%SDS for 5-10 minutes.

Reverse Transcription : Oligonucleotides used for the primer extension were as given below:

RP51A-27 : 5' GTATGACTTTATTGCGCATGTCGACTC 3'

Actin XhoI : 5' CTCTCGAGCAATTGGGACCGTGC 3'

The primer oligonucleotides (20ng) were 5' end labeled using gamma ^{32}P ATP (crude 7000Ci/mM) and T-4 polynucleotide kinase. The labeled oligonucleotides were purified on DE-52 ion exchange column, and ethanol precipitated. 20-25 micrograms of total RNA was used in each primer extension with ~50,000 cpm of the primer in 50 mM Tris HCl pH8, 150 mM KCl, 0.5 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$. Annealing was done by heating the RNA, primer and buffer at 90°C for two minutes followed by slow cooling to room temperature. Fifteen to twenty units of AMV reverse transcriptase and 0.5 mM of each deoxynucleotide was added and the extensions done at 42°C for 45 minutes. The extension products were separated on 8 or 10% polyacrylamide gels containing 8M urea. In cases where dideoxy sequencing was done, a clone of actin in M13 was used as the template with the actin oligonucleotide as a primer. The sequencing reactions were performed with a kit from Pharmacia.

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TABLE 1

Complementation group	Number of alleles	Splicing defect	Transcripts tested
prp (*rna*)2	6	pre-mRNA	Actin
prp (*rna*)3	7	pre-mRNA	Actin
prp (*rna*)6	6	pre-mRNA	Actin
prp (*rna*)9	2	pre-mRNA	Actin
prp (*rna*)17 *(ts 365, ts 487)*	1	Lariat-intermediate	Actin, RP51A
prp (*rna*)18 *(ts 452, ts 503, ts 491)*	3	Lariat-intermediate	Actin, RP51A RP59
prp (*rna*)19 *(ts 89)*	1	Lariat-intermediate	Actin, RP51A
prp (*rna*)20 *(ts 319)*	1	pre-mRNA and transcript larger than pre-mRNA	Actin, RP59
prp (*rna*)21 *(ts 47)*	1	pre-mRNA	Actin, RP51A RP59
prp (*rna*)22 *(ts 89)*	1	pre-mRNA and intron	Actin, RP51A RP59
prp (*rna*)23 *(ts 514)*	1	pre-mRNA	Actin, RP51A RP59
prp (*rna*)24 *(ts 344)*	1	pre-mRNA	Actin, RP51A
prp (*rna*)25 *(ts 449)*	1[a]	pre-mRNA	Actin, RP51A RP59
prp (*rna*)26 *(ts 278, ts 391) (ts 45)*	3[a]	intron	Actin, RP51A CYH2
prp (*rna*)27 *(ts 397)*	1	intron	Actin, RP51A

a --- None of the alleles are *ts*

TABLE 2: LIST OF YEAST STRAINS

Strain	Genotype
SS328	*MATα ade2-101 his3Δ200 lys2-801 ura3-52*
SS330	*MATa ade2-101 his3Δ200 tyr1 ura3-52*
rna1-1	*MATa prp(rna)1-1 ade1 ade2 his7 tyr1 ura1*
RL 92	*MATa prp(rna)2 leu2-3 leu2-112 ura3-52*
SPJ 3.33	*MATa prp(rna)3 his3 leu2 lys2 ura3-52*
RL144	*MATa prp(rna)4 his3Δ–1 leu2-3 leu2-112 lys2 tyr1 ura3-52*
RL173	*MATa prp(rna)5 ade his7 leu2 lys2*
rna 6-1	*MATa prp(rna)6-1 ade1 ade2 his7 lys2 tyr1 ura1*
rna 7-1	*MATa prp(rna)7-1 ade1 ade2 his7 lys2 tyr1 ura1*
rna 8-1	*MATa prp(rna)8-1 ade1 ade2 his7 lys2 tyr1 ura1*
rna 9-1	*MATa prp(rna)9 -1 ade1 ade2 his7 lys2 tyr1 ura1*
SPJ 11.4	*MATa prp(rna)11 his4-512 leu2 ura3-52*
*ts*136#7A	*MATα prp(rna)1 ade his met trp ura*
SS304	*MATα prp(rna)2 ade2-1 his3-532 trp1-289 ura3-1 ura3-2*
rna3-3A	*MATα prp(rna)3 ade leu*
SPJ 4.43	*MATα prp(rna)4 ade1 ade2 his7 leu2 lys2 ura3-52*
SPJ 5.41	*MATα prp(rna)5 his3 his7 leu2 ura3-52*
SPJ 6.68	*MATα prp(rna)6 his3 lys2 ura3-52*
rna7 (AH)	*MATα prp(rna)7 ade arg leu ura1*
rna8 (AH)	*MATα prp(rna)8 ade arg trp ura1*
SPJ 10.2	*MATα prp(rna)11 ade his leu lys tyr ura*

FIGURE LEGENDS

Fig. 1A. Scheme used for the isolation of splicing mutants.

Fig. 1B. A representative northern blot from a primary screen of twenty five *ts* mutants. 10μg of total RNA from each mutant strain, after two hours at non-permissive temperature, was run on a formaldehyde-agarose gel and then blotted. The probe used was a labeled fragment of the actin intron. The last lane had total RNA from *prp* (*rna*) 2, a strain known to accumulate pre-mRNAs.

A

EMS mutagenesis of yeast

↓

Generation of a bank of temperature sensitive strains

↓

Preparation of total RNA from *ts* strains at non-permissive temperature

↓

Probing of northern blots with actin intron sequences

↓

Reanalysis of candidates with splicing defects at permissive and non-permissive temperature

↓

Complementation and segregation analysis

B

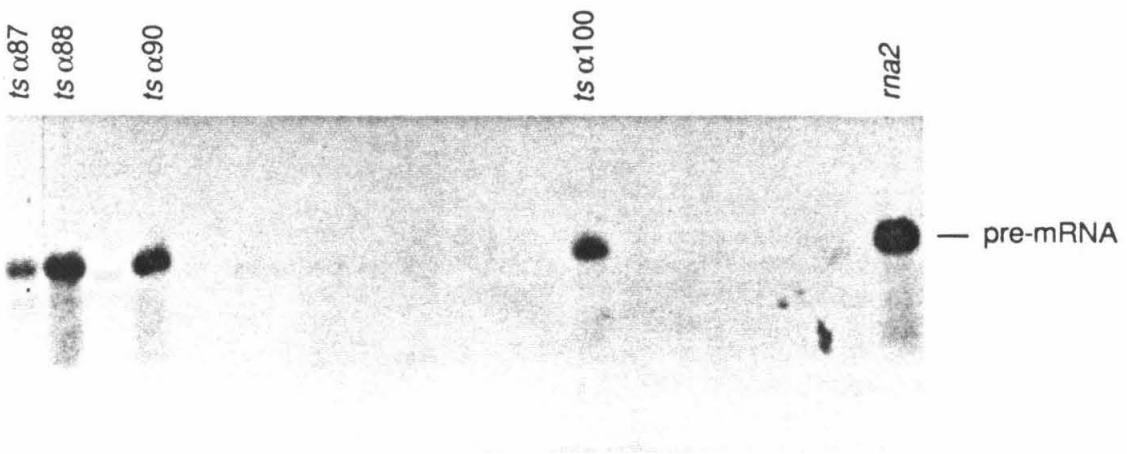


Fig. 2 Re-screening, at both permissive and non-permissive temperatures, of some positives obtained from the primary screen. For each candidate 10μg of total RNA from cells at 23°C or after a two hour shift to non-permissive temperature (37°C) was analyzed. RNA from wild type cells and from *prp* (*rna*)5 at both temperatures was also analyzed as controls. The blots were probed in panel A with a fragment of the actin intron and in panel B with a probe for the detection of spliced actin mRNA.

A
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
— intron
— pre-mRNA
a b c d e f g h i j k l m n
ts a278
ts a319
ts a449
ts a503
ts a514
rna5
SS330
B
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
— mRNA
a b c d e f g h i j k l m n
ts a278
ts a319
ts a449
ts a503
ts a514
rna5
SS330

Fig. 3A Reverse transcription using a primer 5' of the RP51A branch point. 20μg of total RNA prepared from each of the four different strains denoted above the gel (SS330, *ts* 45, *ts* 503, *ts* 514) was used for primer extension. RNA from cells at both permissive and non-permissive temperature was analysed as indicated at the bottom of the gel. Schematic representations of the two classes of primer extension stops corresponding to 5' ends of lariat molecules, or 5' ends of the pre-mRNA are also made.

3B. Reverse transcription from a primer 5' of the actin branch point. 20μg of total RNA from the wild type SS330 strain , the mutant *ts* 391 accumulating the released intron, and the strain *ts* 503 that accumulates the lariat intermediates was used. A schematic representation of the primer extension stop is drawn.

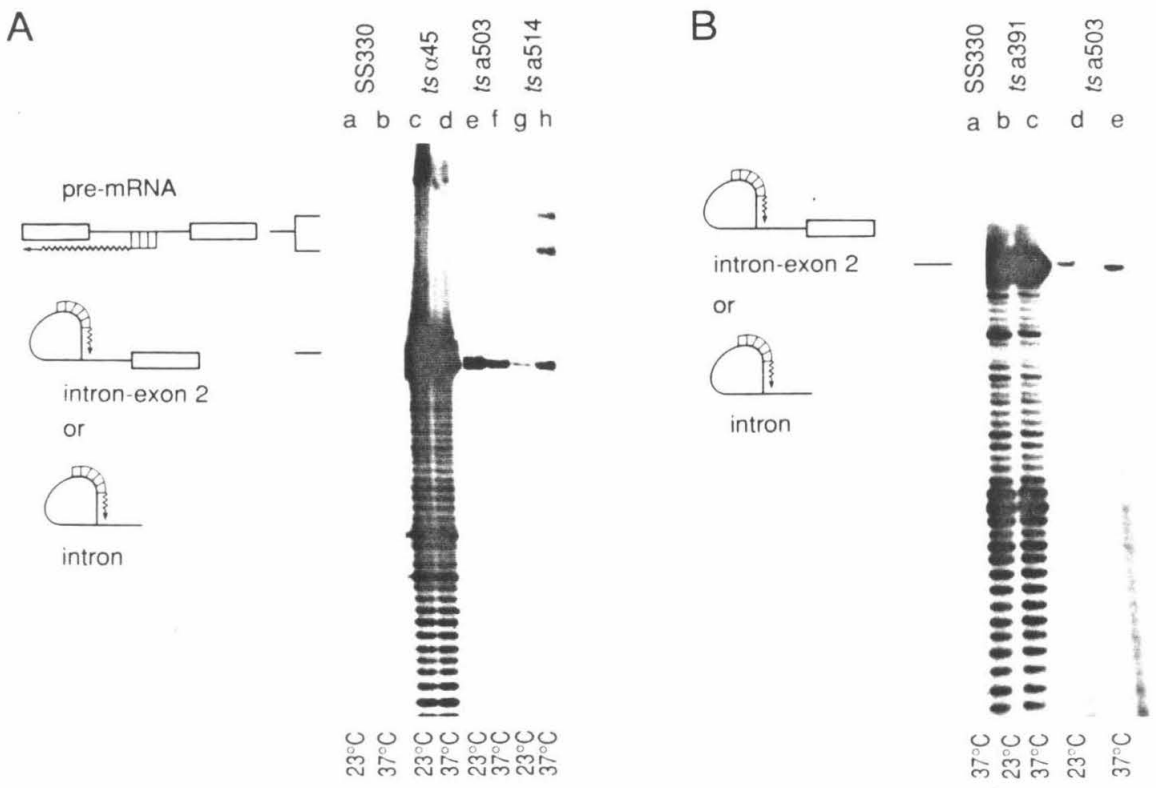

A
SS330
ts α45
ts a503
ts a514
a b c d e f g h
pre-mRNA
intron-exon 2
or
intron
23°C
37°C
23°C
37°C
23°C
37°C
23°C
37°C
B
SS330
ts a391
ts a503
a b c d e
intron-exon 2
or
intron
37°C
23°C
37°C
23°C
37°C

Fig. 4. Complementation analysis of *prp* mutants. Diploids generated from all pairwise crosses of *ts* 47, *ts* 87, *ts* 503, *ts* 514, *prp* (*rna*)2 and the wild type strain. The left plate shows growth at permissive temperature (23°C) and the right plate the growth of the same diploids at non-permissive temperature (37°C). Non-complementing diploids exhibit no growth at 37°C.

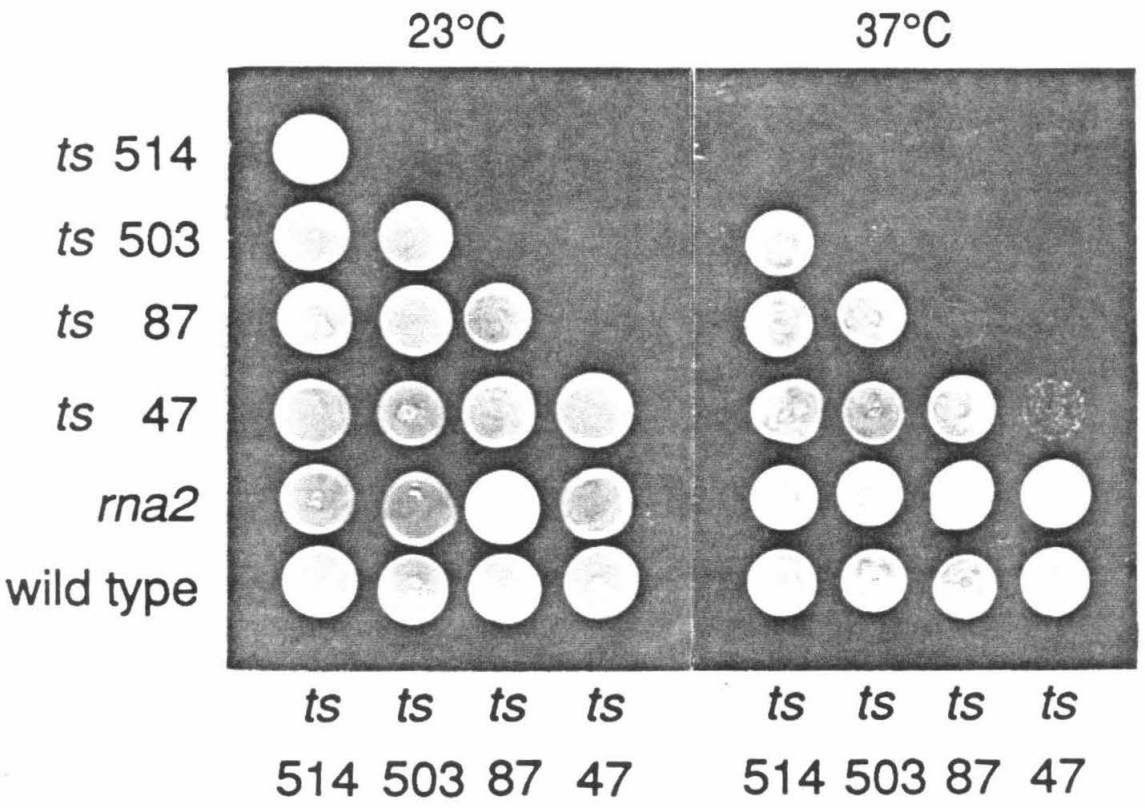
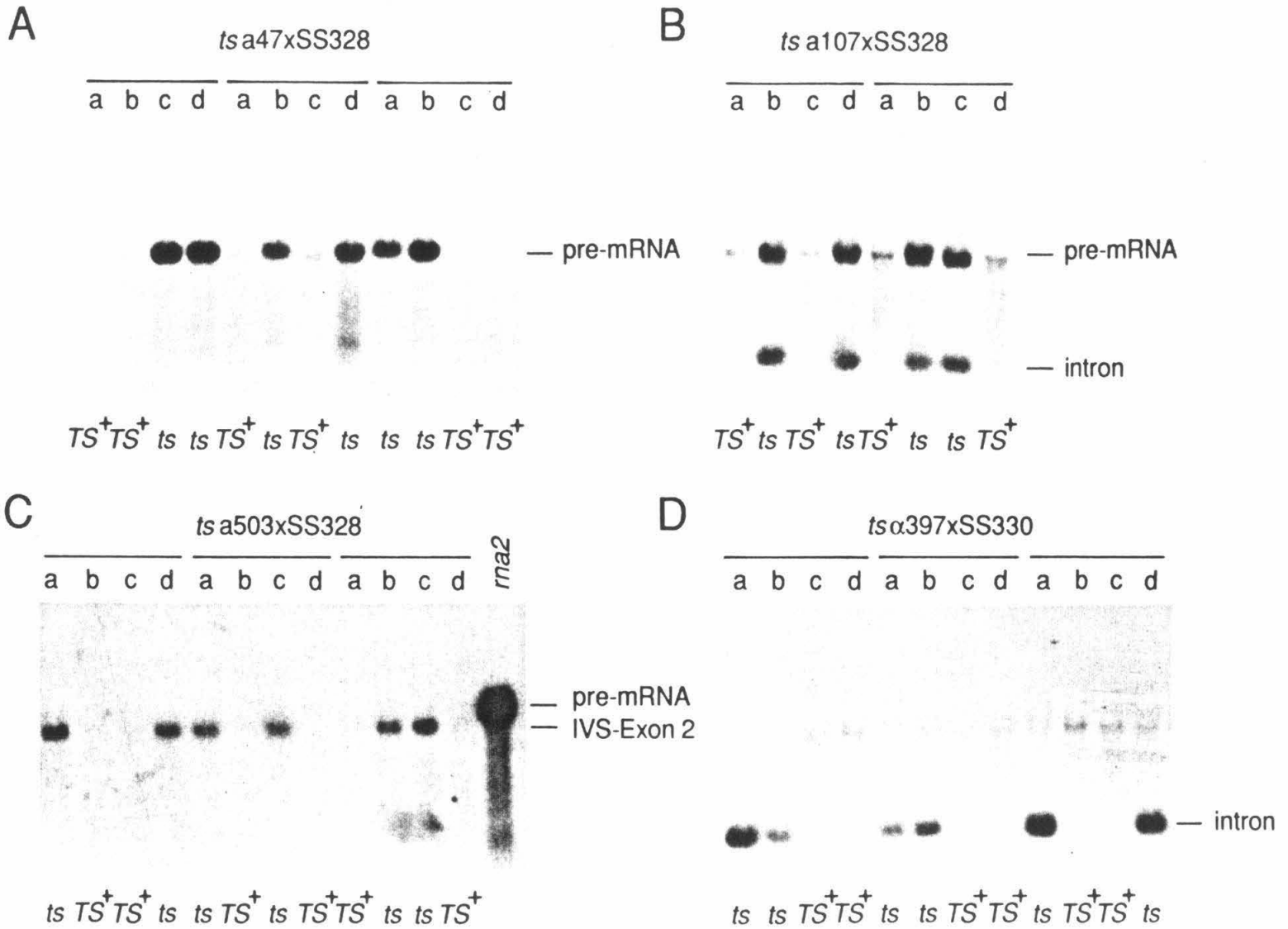

23°C
37°C
ts 514
ts 503
ts 87
ts 47
rna2
wild type
ts 514
ts 503
ts 87
ts 47
ts 514
ts 503
ts 87
ts 47

Fig. 5 Co-segregation of the temperature sensitive and splicing phenotypes. An example from each of the four phenotypic classes of splicing mutants was analyzed. Heterozygous diploids were sporulated and tetrads dissected in each case. Spores from several tetrads were grown to log phase and shifted to non-permissive temperature for two hours before RNA extraction. The RNAs were assayed on northern blots for the splicing phenotype. Panel A: pre-mRNA accumulation, panel B: pre-mRNA and intron accumulation, panel C: lariat intermediate accumulation (IVS-Exon2) and panel D: intron accumulation. The growth phenotype of the spores at 37°C is also indicated in the figure.


A
ts a47xSS328
a b c d a b c d a b c d
— pre-mRNA
TS+TS+ ts ts TS+ ts TS+ ts ts ts TS+TS+
B
ts a107xSS328
a b c d a b c d
— pre-mRNA
— intron
TS+ ts TS+ ts TS+ ts ts TS+
C
ts a503xSS328
a b c d a b c d a b c d
rna2
— pre-mRNA
— IVS-Exon 2
ts TS+TS+ ts ts TS+ ts TS+TS+ ts ts TS+
D
ts α397xSS330
a b c d a b c d a b c d
— intron
ts ts TS+TS+ ts ts TS+TS+ ts TS+TS+ ts

CHAPTER 3

PRP 18 : A gene product required for the intron-exon2 cleavage and ligation of exons

ABSTRACT

We have investigated the role of a novel temperature sensitive splicing mutation *prp18* . We have previously shown that at the restrictive temperature *in vivo* an accumulation of the lariat intermediate of splicing occurs. We have now used the yeast *in vitro* splicing system to demonstrate that extracts from this mutant strain are heat labile for the second reaction of splicing. The heat inactivation of *prp18* extracts results from loss of activity of an exchangeable component. Inactivated *prp18* extracts are complemented by heat-inactivated extracts from other mutants or by fractions from wild type extracts. In heat inactivated *prp18* extracts, 40S splicing complexes containing lariat intermediate and exon1 can assemble. The intermediates in this 40S complex can be chased to products by complementing extracts in the presence of ATP. Both complementation of extracts and chasing of the isolated *prp18* spliceosomes takes place with micrococcal nuclease-treated extracts. Furthermore, the complementation profile with fractions of wild type extracts indicates that the splicing defect results from a mutation in a previously unidentified factor required for the second step of splicing. The isolation of this mutant as a temperature sensitive lethal has also facilitated cloning of the wild type allele by complementation.

INTRODUCTION

The recognition and splice site selection in nuclear pre-mRNA splicing is dictated by three short stretches of sequence elements in the intron (Shapiro and Senapathy 1987). The specificity of splice junction selection and the juxtaposition of the exons is achieved by the recognition of these cis-acting conserved elements in the intron by cellular factors that include proteins and small nuclear ribonucleoprotein particles (snRNPs) (reviewed in Sharp, 1987; Maniatis and Reed 1987). The development of *in vitro* splicing systems has led to considerable progress in the elucidation of the pathway of nuclear pre-mRNA splicing (reviewed in Padgett *et al.*, 1986; Green 1986). The pre-mRNA is spliced in a large multicomponent structure termed the spliceosome (Brody and Abelson 1985; Frendewey and

Keller 1985; Grabowski *et al.*, 1985; Bindereif and Green 1986; Perkins *et al.*, 1986). Splicing reactions that take place in the spliceosome occur *via* a two-step mechanism. First the pre-mRNA is cleaved at the 5' splice junction and this results in the formation of a lariat intron-exon2 structure in which the first base of the intron is covalently linked to a specific adenosine near the 3' splice site. The cleaved exon1 and the lariat intron-exon2 are the intermediates of the splicing reaction. The second reaction involves cleavage at the 3' splice site and the ligation of the two exons to produce the mature mRNA and the lariat intron.

The complexity of the splicing reaction and the large particle within which the reactions take place suggests the need for multiple functions. It is now clear that at least five snRNPs (U1, U2, U4, U5 and U6), are involved in the splicing of both yeast and higher eukaryotic pre-mRNA (Steitz *et al.*, 1987; Guthrie and Patterson 1988). These snRNPs have been found to associate with active splicing complexes. The assembly intermediates are resolved on non-denaturing gels and they vary in the order of appearance and their snRNP composition. U1 binds to the pre-mRNA first and this is followed by the binding of U2, U4, U5 and U6 snRNPs (Konarska and Sharp 1986; Pikielny *et al.*, 1986; Cheng and Abelson 1987; Bindereif and Green 1987; Ruby and Abelson 1988). The requirement in splicing for protein factors, apart from snRNPs, has been demonstrated through biochemical fractionation of splicing extracts (Krainer and Maniatis 1985; Cheng and Abelson 1986; Kraemer *et al.*, 1987). A role for hnRNP proteins in splicing is also indicated from inhibition experiments performed with antibodies against hnRNP core proteins (Choi *et al.*, 1986; Sierakowska *et al.*, 1986).

Analysis of splicing factors in yeast can readily employ a combination of biochemical, classical genetic and molecular approaches. Such an approach has taken advantage of the availability of a set of temperature sensitive mutations (*rna2* to*11*) that affect pre-mRNA splicing *in vivo* (reviewed in Warner 1987; Vijayraghavan and Abelson 1988). Henceforth all the *rna* mutants will be called as pre-RNA processing: *prp* mutants. Temperature sensitive strains bearing these mutations when shifted to non-permissive tempera-

tures accumulate pre-mRNAs (Rosbash *et al.*, 1981; Miller 1984). These mutations were the best candidates for the genetic analysis of yeast pre-mRNA splicing components. *In vitro* heat inactivation of splicing extracts from many of these *prp* complementation groups has implied a more direct role for these gene products in pre-mRNA splicing. Consistent with the *in vivo* observation of pre-mRNA accumulation at non-permissive temperature in all of the *prp 2- 11* strains, inactivation of both steps of splicing was observed upon heating of splicing extracts from several groups (Lustig *et al.*, 1986). In addition, most of the *PRP* gene products have been demonstrated to be required for spliceosome formation itself (Lin *et al.*, 1987). The *PRP2* gene product acts after spliceosome assembly and is required for the first splicing reaction. Studies on the role of *PRP2* in *in vitro* splicing systems have proposed the need for several extrinsic and intrinsic factors for both splicing reactions (Lin *et al.*, 1987). Apart from the *in vitro* characterization of the *prp 2-11* mutants, many of these genes have been cloned and their molecular characterization has been initiated (Last *et al.*, 1984; Lee *et al.*, 1984; Soltyk *et al.*, 1984; Jackson *et al.*, 1988; reviewed in Vijayraghavan and Abelson 1988). Antibodies directed against *PRP8* and *PRP11* proteins inhibit *in vitro* splicing reactions, reaffirming their essential role in splicing reactions (Jackson *et al.*, 1988; Chang *et al.*, 1988). The *PRP8* protein has been demonstrated to associate with U5 snRNA (Lossky *et al.*, 1987). The *PRP11* protein associates with spliceosomes and is also present in a pre-assembled 30S complex. Further analysis of the *PRP* gene products is likely to reveal their precise role in the splicing machinery and determine if they are homologs of protein factors required in higher eukaryotic spliceosomes. Thus a combined genetic and biochemical approach has proved successful for the analysis of yeast splicing factors.

The isolation of several new temperature sensitive mutations affecting pre-mRNA processing has been done (Vijayraghavan, Company and Abelson manuscript submitted). Isolation of these eleven new complementation groups affecting pre-mRNA splicing *in vivo* , was done by a direct search for such mutants. Interestingly, several groups (4) with

novel phenotypes were obtained, which specifically affect late stages of splicing : cleavage at the 3' splice site and exon ligation, and degradation of the lariat intron. These mutants facilitate the development of assays for specific steps in splicing, and therefore the analysis and purification of specific splicing factors. The isolation of two complementation groups (*prp17* and *prp18*) with the phenotype of lariat intermediate accumulation allows an opportunity for the analysis of splicing components required for the second reaction of splicing. In these mutants the lariat accumulation results from a specific defect affecting the second reaction of splicing, as no pre-mRNA is observed in these strains. Fractionation of wild type yeast splicing extracts has identified one fraction needed specifically for the second step of splicing (Cheng and Abelson 1986). Studies on the *prp2* heat inactivated spliceosomes have proposed the requirement for a second set of factors in the second splicing reaction (Lin *et al.*, 1987). The need for factors involved only in cleavage at the 3' splice site and exon ligation has also been evident in mammalian splicing systems. At least two factors have been reported to be required at this stage of splicing in higher eukaryotic splicing systems (Krainer and Maniatis 1985; Kraemer *et al.*, 1987).

A combination of biochemical and molecular analysis has been initiated for study of the role of *PRP18* in splicing. We report that a mutation in one of the previously hypothesized yeast splicing factors results in lariat intermediate accumulation. A specific requirement for ATP in the second step of splicing is also observed. The isolation of this novel mutation as temperature sensitive lethal, allowed the cloning of the wild type *PRP18* locus.

RESULTS

Heat inactivation of splicing extracts from prp18

The isolation of a *ts* mutation affecting the second step of splicing *in vivo* provides a method for the study of factors required in the late stages of splicing. We have applied the *in vitro* yeast splicing system and the heat inactivation of mutant extracts for the study of the role of *PRP18* in splicing. Whole cell splicing extracts capable of *in vitro* splicing

reactions have been prepared from strains bearing the *prp18* mutation. Extracts from cells grown at permissive temperature were assayed for splicing activity at permissive temperature *in vitro* (15°C). The splicing activity is comparable to that of wild type extracts. The first step of splicing seems unaffected; however, the extent of the second reaction of splicing in different *prp18* extracts is variable (Fig. 1A compares activities in *prp18-2* strains, 503-1a and 503-6c). Hence, the efficiency of cleavage at the 3' splice site and the ligation of the exons appears to be affected in whole cell extracts prepared from this mutant strain.

To investigate the function of the *PRP18* gene product, heat inactivation of extracts from *prp18-2* strain (*ts* 503-6c) was carried out . Inactivation of extracts was done by heating for 15 to 60 min. at 30-32°C under conditions described in Materials and Methods. The heat inactivated extracts were assayed at permissive temperature (15°C for 60 min.). In extracts from *prp18* , lariat intermediate and exon1 accumulate and there is a substantial decrease in the levels of the mRNA and the lariat intron after 60min. of heat inactivation (Fig. 1B). Similar accumulation of lariat intermediate is observed *in vivo* for chromosomal transcripts from the actin, RP51 and RP59, genes after shift to non-permissive conditions (Vijayraghavan, Company and Abelson; manuscript submitted). Some decrease in the efficiency of the first splicing reaction is observed upon prolonged incubation of *prp18* extracts at 30-32°C; the cause is not known. However, heat treatment of some wild type extracts also causes a reduced efficiency of total splicing activity (Lustig *et al.*, 1986, this study data not presented).

In vitro complementation of inactivated extracts

Splicing extracts prepared from *prp18* strains accumulate lariat intermediate and exon1. One possible reason for this effect is denaturation or inactivation of a specific splicing component, as a result of temperature sensitivity of the protein or RNA. To substantiate this theory, inactivated *prp18* extracts were used to complement inactivated extracts from

other *ts* strains. Complete inactivation of extracts from *prp5* or *prp11* strains was obtained with previously described conditions (Lustig *et al.*, 1986). Mixing of inactivated *prp18* extract with inactivated *prp5* or inactivated *prp11* extract, resulted in restoration of splicing activity (Fig. 2, lanes 1 and 2). A combination of *ts* 503-6c extract with inactivated extracts from cells bearing the same *prp18* mutation in a different genetic background did not result in complementation (Fig. 2, lane 6). Thus, the loss of activity is due, at least in part, to inactivation of the *PRP18* gene product; the complementation also indicates that some of the splicing factors can exchange under these assay conditions.

The complementation *in vitro* with *prp5* or *prp11* inactivated extracts indicated that complementation with the *PRP18* wild type gene product should bepossible. To demonstrate this we have used fractions of wild type extracts, *i.e.*, Fraction I (40P3) and Fractions II plus III (40W) obtained by ammonium sulfate precipitation (Cheng and Abelson 1986). Complementation of *prp18* splicing defect is striking with 40P3 (fraction I) (Fig. 2, lane 3), while 40W does not seem to contain much complementing activity (Fig.2, lane 5). We also tested whether *prp18* inactivated extract could be complemented by micrococcal nuclease-treated wild type extracts. Micrococcal nuclease treatment of wild type extracts causes a complete loss of splicing activity (data not shown), as has been previously reported (Cheng and Abelson 1986). Mixing of the treated wild type extract and the inactivated *prp18* extract restores splicing activity (Fig. 2, lane 5). Therefore, complementation of inactivated *prp18* extracts does not require the presence of intact snRNPs, although the complementing activity resides in an snRNP-enriched fraction (40P3).

Isolation of heat inactivated prp18 spliceosomes and chasing of the intermediates of splicing

It has been demonstrated by various means that the first step of splicing takes place only after the assembly of the spliceosome is complete (Cheng and Abelson 1987; Lin *et al.*, 1987; Pikielny *et al.*, 1986; Konarska and Sharp 1987). Complementation with inactivated

crude extracts provides evidence for exchangeability of specific complementing factors. However, these experiments do not address whether exchange of the factors occurs only before or after spliceosome assembly. Isolation of *prp18* heat inactivated spliceosome will allow the testing of the exchangeability of complementing factors after assembly, and the determination of whether this isolated complex is an intermediate in splicing.

Large scale splicing reactions performed with heat inactivated *prp18* extract containing pre-mRNA lariat intermediate and exon1, were sedimented through glycerol gradients; fractions were collected and counted (Fig. 3A). The labeled pre-mRNA across the 40S and the 30S region of the gradient was monitored on denaturing gels, revealing a peak of lariat intermediate, exon1 and pre-mRNA in the 40S region of the gradient . This indicates that the mutant spliceosomes remain intact after inactivation and gradient sedimentation. Upon addition of inactivated *prp5* or *prp11* extracts, or the ammonium sulfate fraction 40P3 to the spliceosomal complexes in fraction 12, a conversion to products is observed (Fig. 3B , lanes 1, 2, 3). No products are formed in the absence of complementing extract (Fig. 3B, lane 7). The chasing of the spliceosomal intermediates is not complete, implying some inefficiency in complementation after assembly of the spliceosome. Complementation of the inactivated spliceosome has a requirement for ATP, apart from the need for the requisite complementing extract (Fig.3B , lane 8). This experiment demonstrates the need for ATP in the second step of the splicing reaction.

The chasing upon addition of complementing fractions could be due to 1) chasing of the lariat intermediate and exon1 in the preformed complex or 2) dis-assembly and re-assembly of splicing components in the spliceosome and the complementing fraction or 3) the chasing of the pre-mRNA in this region of the gradient. In all cases where chasing is observed, a decrease in the level of lariat intermediate accompanies the appearance of intron and mRNA. This supports our belief that chasing of the spliceosomal intermediates takes place. The second possibility has been ruled out by the addition of a second transcript during chasing reactions that, if spliced, would result in a different sized mRNA. These

chasing reactions did not result in any mRNA production from the second transcript (data not shown). In addition the above experiment also shows that if pre-mRNA from this region of the gradient is being chased, it must be a form of assembled spliceosome. We cannot rule out this possibility.

As with the complementation, chasing of splicing intermediates is obtained with micrococcal nuclease-treated crude extracts or ammonium sulfate fraction 40P3 (Fig. 3B, lanes 4 and 5), again suggesting that neither complementation nor chasing requires intact snRNPs. The demonstration of the first step of splicing in inactivated *prp18* extracts indicates that *prp18* function is probably not needed for the formation of spliceosomes but is required for the second step of splicing.

Isolation of PRP18 gene

Molecular analysis of the locus encoding the *PRP18* gene product is essential for the complete analysis of the role of this gene product in splicing. The tight linkage of the lariat accumulation phenotype with the *ts* lethality in the *prp18* strains (Vijayraghavan, Company and Abelson; manuscript submitted) allows cloning of the wild type gene product. The wild type allele of *prp18* was cloned on the basis of its ability to complement the *ts* mutation in the *PRP18* gene. A *prp18* strain was transformed with a yeast genomic library constructed in a yeast centromere plasmid library, and incubated on selective plates at 23C. The resulting *URA* transformant colonies were replica plated to pre-warmed rich plates (YPD) at 37°C to select temperature resistant colonies. Yeast plasmids were recovered from several of these colonies and transferred to *E. coli* . The plasmids were propagated in *E. coli* and preliminary restriction mapping indicated that the recombinant plasmids were substantially identical. Retransformation of the *prp18* strain with these plasmids from the yeast library, after propagation in *E. coli* , reaffirmed that the presence of the clone confers temperature resistance. One of these plasmids (pUV18) was used in further experiments. Transformation of the yeast vector YCp50 did not confer wild type growth.

Strains bearing the plasmid pUV18 were patched on selective plates containing 5-Floro-orotic acid, to select for the loss of centromere plasmid from the transformants. *ura* colonies obtained by this method were tested for temperature sensitivity, and were found to have reverted to *ts* phenotype. Thus, the presence of the plasmid alone confers a wild type phenotype. Transformation of the clone in a *ts prp* strain other the *prp18* does not result in complementation of other mutation. All of these results indicate that the plasmid suppresses the *prp18 ts* phenotype specifically.

pUV18 contained a yeast genomic DNA of about 7kb. A restriction map was generated. To define the limits of the complementing sequence, specific restriction fragments of the insert were subcloned into a yeast low copy (CEN) shuttle plasmid (pPHY18). Fig. 4 shows that while plasmids pUV18-2, pUV18-4 and pUV18-20, when transformed into *prp18* are capable of generating *URA,* temperature resistant colonies and hence complementing the *prp18* mutation; plasmids pUV18-3, pUV18-17, pUV18-18 and pUV18-19 yield *URA* colonies that are temperature sensitive. These results imply that the complementing sequence lies in the BamHI to NruI region of the initially isolated genomic fragment in pUV18. Subclone pUV18-20 that was capable of complementing the *ts* phenotype was used in a second series of subcloning experiments and several restriction fragments were cloned into the CEN vector pPHY18. These transformation experiments indicated that the HindIII site interrupts the complementing sequence, but leave unclear if the XhoI site lies within or outside the complementing sequence (Fig. 4). The complementing fragment (region from BamHI to NruI) was also cloned into a yeast multicopy vector (2μ *i. e.* pSEY18) in addition to the low copy vectors used above. The increased expression of this genomic fragment on this plasmid again gave complete complementation. Thus, complementation works effectively at either single or higher copy number yeast plasmids.

Complementation of the mutant splicing phenotype

prp18 strain accumulates lariat intermediates of splicing *in vivo* at both permissive and non-permissive temperatures with no increase in the levels of pre-mRNA (Vijayraghavan,

Company and Abelson; manuscript submitted). Total RNA was prepared from the original *prp18* strain, the *prp18* strains transformed with complementing plasmids (pUV18 and pUV18-15), and from the strain transformed with the vector YCp50. The RNA was run on formaldehyde agarose gels, blotted to nylon to membrane and probed with a labelled fragment of the actin intron. Fig.5A, lanes 7 and 8 show accumulation of lariat intermediate of actin in the original *prp18* strain and in the *prp18* strain bearing only the YCp50 vector (lanes 1 and 2) at both permissive and non-permissive temperatures. No accumulation of lariat intermediate takes place in the strain containing the original complementing clone pUV18 (lanes 3 and 4) or the subclone pUV18-15 (lanes 5 and 6). Reverse transcription experiments using a primer complementary to the RP51 intron were done (Fig. 5b). These experiments also confirm that the levels of lariat intermediate of RP51A are reduced to the wild type levels.

Cloned DNA maps to the PRP18 locus

To prove that the complementing clone obtained corresponds to *PRP18* gene and is not an extragenic suppressor capable of relieving the *prp* phenotype, integration of the cloned complementing genomic DNA was performed. A complementing fragment (the 5kb RI fragment cloned in pUV18-4) was cloned into the yeast integrating plasmid (YIP5). Integration into the yeast genome was done after linearization of this YIP5 subclone at the unique XhoI site in the genomic fragment (Fig 6A). *URA* colonies were selected for after transformation of the linearized DNA into the wild type temperature resistant strain SS330. The *URA* strains were crossed to the temperature sensitive *ura, prp18* strain, and diploids were selected for prototrophy. Sporulation of diploids and dissection of spores from two diploid clones demonstrated a co-segregation of the *TS* and *URA* phenotype and of the *ts* and *ura* phenotype in several tetrads (fourteen from each diploid). This analysis shows an integration of the cloned DNA with the homologous chromosomal *PRP18* locus.

Southern analysis was also performed as a second means of proving homologous recombination to the *PRP18* locus. Genomic DNA preparations were done from the un-

transformed strains SS330 and *prp18* , two SS330 *URA* integrative transformants, and the diploid of integrative transformant crossed to *prp18* . All the genomic DNA preparations were digested with the enzyme SalI. The DNA was then run on agarose gels, and blotted and probed with a fragment containing the entire complementing region. Fig. 6A cartoons the expected integration event to the *PRP18* locus. As expected the probe detects only one SalI fragment (Fig. 6B) in the untransformed parental strains (no SalI in the *PRP18* gene). Each integration event brings in one SalI site from the YIP5 vector sequences. Hence, as a result of integration the probe hybridized to two fragments in the haploid *URA* integrant SS330 Ib (Fig.6B, lane 4). Three fragments are detected in the SS330 integrative transformant -Ia (Fig.6B, lane 3), an event most likely due to tandem insertion of two copies of the cloned DNA. All of the integration, segregation and southern blot analysis is consistent with our having cloned the wild type allele of *PRP18* .

Identification of the PRP18 transcript

The cloning of the *PRP18* DNA allows its use as a probe in RNA blots, for determination of the transcription units on the complementing genomic DNA fragment. This analysis is likely to identify the *PRP18* transcript. Total RNA was prepared from wild type cells at both 23C and 37C. Selection of poly A^+ RNA was done by an oligo-dT cellulose column. A formaldehyde agarose gel was run with both total RNA (10 and 20 μg) and polyA^+ enriched RNA (2 μg), and blotted to nylon membrane. The use of the XhoI to HindIII (~1.1 kb) fragment as a probe on this blot detects two transcripts of ~ 1.5kb and ~2.3 kb in length respectively (Fig7A). The HindIII to BamHI DNA fragment (~0.7kb), when used as a probe, detected two RNAs of ~2.3 kb and ~1.8 kb (Fig. 7B). The ~2.3 kb poly A^+ RNA therefore spans the HindIII site, which has been shown by complementation experiments to lie within the complementing region (Fig. 4). These blots make reasonable the deduction that the ~2.3 kb polyA^+ RNA is being transcribed from the *RNA18* locus. The ~1.8 kb and the ~1.5kb transcripts derive from flanking genomic sequences. *In vitro* complementation with micrococcal nuclease-treated extracts and the detection of a polyA^+ RNA

being transcribed from the *PRP18* locus both indicate that the gene product is a protein component of the splicing apparatus.

DISCUSSION

Identification of a factor affecting late stages of splicing

Biochemical fractionation of splicing extracts and analysis of the assembly of the spliceosomes has indicated the importance of snRNP and protein factors in splicing (reviewed in Sharp 1987; Maniatis and Reed 1987). In the study of pre-mRNA splicing in yeast a combined genetic and biochemical approach has been taken with the characterization of the *PRP2-11* gene products (Warner 1987; Vijayraghavan and Abelson 1988). We have recently reported the generation of a bank of temperature sensitive mutations, which was screened for defects in pre-mRNA splicing (Vijayraghavan, Company and Abelson, manuscript submitted). One of the complementation groups with a novel splicing phenotype displayed an accumulation, *in vivo* , of the lariat intermediate. This phenotype resulted from a trans-acting defect affecting splicing and thus provided a means for the analysis of the components required specifically for the second step of splicing. In this paper we have investigated the role of the *PRP18* gene product *in vitro* and have also obtained some molecular information about the *PRP18* gene.

Splicing extracts prepared from the mutant strain are defective for the second splicing reaction, and accumulated the lariat intermediate and exon1. This splicing defect after heat inactivation of splicing extracts is the same as the *in vivo* phenotype after shift to non-permissive temperature (Vijayraghavan, Company and Abelson, manuscript submitted). The loss of activity for the 3' intron-exon cleavage and exon ligation could result from specific inactivation of a splicing component, or may be explained as a non-specific effect. The second possibility has to be considered as some loss of splicing activity is observed upon heating of even wild type extracts (Lustig *et al.*, 1986; this study). However this is unlikely on three counts. First, loss of splicing activity in wild type extracts, when ob-

served, always diminishes total splicing activity due to a decrease in both reactions of splicing. Second, the *in vitro* loss of activity for the second step of splicing, observed in *prp18* strains, can be complemented with inactivated extracts from different mutant strains and also with fractions of wild type extracts. Restoration of activity in these mixing experiments probably results from the exchange of active components from each of the inactivated extracts. Third, the cloned *PRP18* gene completely suppresses the *prp18* phenotype. The results strongly imply that splicing defect in *prp18* results from a specific inactivation of a splicing component.

Complementation of heat inactivated *prp18* extracts indicated that complementation could be achieved by addition of wild type fractions containing the *PRP18* gene product. Fractionation of wild type extracts using ammonium sulfate precipitation, followed by heparin agarose chromatography, has already identified three fractions (I, II and III) , necessary for splicing (Cheng and Abelson 1986). Fractions I and II are together capable of assembling spliceosomes and supporting the first step of splicing. Fraction III is required for completion of splicing, in the presence of fractions I and II. Complementation of heat inactivated *prp18* extracts is achieved best with 40P3 (fraction I) and not with 40W (fractions II and III). This indicated to us that there exists a requirement for components other those present in fraction III for the completion of splicing in the heat inactivated *prp18* extracts. The strong complementation obtained with 40P3 (fraction I) implied that the complementing component needed for *prp18* is enriched in wild type fraction I.

PRP18 probably corresponds to one of the as yet unidentified, but previously hypothesized factors c_n . Proposal for the existence of c_n came from experiments involving chasing of *prp2* heat inactivated spliceosomes (Lin *et al.*, 1987). In these studies chasing of heat inactivated *prp2* spliceosomes containing only pre-mRNA with 40P3 (fraction I) resulted in completion of both splicing reactions, while complementation of *prp2* spliceosomes with 40W (fractions II and III) resulted in accumulation of lariat intermediate. These experiments indicated that some factor(s) other than those present in fraction III are re-

quired for the second step of splicing and they were deduced to be enriched in 40P3 (fraction I). The complementation of heat inactivated *prp18* extract by fraction I implies that the mutant function in this case is possibly one of the c_n factors.

Fractionation of HeLa cell extracts has identified splicing factors required for the second step of splicing (Krainer and Maniatis 1985). One component (SF3) identified by complementation assays was described as a heat sensitive factor present in nuclear extracts. In a preliminary fractionation scheme, SF3 co-fractionated with snRNP- containing fractions. The other component required for the second step of splicing (SF4A) was identified in fractions depleted in snRNPs. The role of SF3 and SF4A in splicing reactions is similar to that of *PRP18* . The fractionation of SF3 and the activity complementing the *prp18* defect, in the preliminary analysis, occurs in snRNP-enriched fractions. It is therefore tempting to compare these two factors. Any further analogy must await better experiments, using probes designed specifically for this purpose.

prp18 spliceosomes represent an intermediate in the splicing pathway

The complementation of heat inactivated *prp18* extracts by other inactivated extracts of *prp* strains implies that splicing components can exchange. The formation of the spliceosome takes place even after heat inactivation, suggesting that at least some of the *PRP18* functions are dispensable for spliceosome formation. Experiments were designed to test whether the lariats in the spliceosome could be chased to products; and thus to determine whether these spliceosomes represent intermediates in splicing. Chasing of the intermediates of splicing is achieved, with some inefficiency, in the presence of complementing factors. A strict requirement for ATP is observed for chasing. As with complementation, chasing is also observed with micrococcal nuclease-treated crude extracts, indicating the dispensability of intact snRNPs for complementation. This still does not rule out the fact that *PRP18* is a protein component of snRNP(s), or a dissociable part of a multicomponent

structure. This component must be exchangeable with the mutant protein even after assembly of the spliceosome.

The experiments with the chasing of the *prp18* spliceosome reaffirm the need for the *PRP18* gene product for the completion of splicing. It is still unclear if *prp18* is easily dissociated from the splicing complexes or if it exists as a component of the inactivated spliceosomes. The fact that all factors needed for splicing are not present in gradient isolated spliceosomes has been noted in earlier studies. Spliceosomes containing only pre-mRNA, when isolated from gradients, are incapable of chasing the pre-mRNA without the addition of splicing components and ATP (Grabowski *et al.*, 1985). The incomplete nature of spliceosomes obtained from *prp2* heat inactivated extracts has also been reported (Lin *et al.*, 1987). The incomplete nature of *prp2* or *prp18* heat inactivated spliceosomes might result from a loss of factors upon heat inactivation and/or gradient sedimentation.

Multiple roles for ATP in splicing have been observed for the assembly of the spliceosome and for the first reaction of splicing (Cheng and Abelson 1987; Lin *et al.*, 1987; Konarska and Sharp 1987; Pikielny *et al.*, 1986). The requirement for ATP in the final step of splicing, 3' cleavage and exon ligation has not been defined. We have shown a requirement for ATP in the chasing reactions with *prp18* spliceosomes. In addition we have found that only chasing of the preassembled complexes takes place. Since the gradient fractions that contain the intermediates most probably also have assembly intermediates with pre-mRNA we cannot exclude the possibility that this assembled pre-mRNA is being chased. The correlation between the appearance of mRNA and the decrease in the lariat intermediate, supports our belief that the chasing of the intermediates occurs. Recent experiments with HeLa extracts have reported a requirement for ATP for the second reaction of splicing (Sawa *et al.*, 1988). In these experiments the second splicing reaction was selectively inhibited and completion of splicing in such extracts occurred only in the presence of ATP and additional nuclear extract. However, the authors do not address the possibility of chasing pre-mRNA in the isolated complex to product, or a possible ATP requirement for

the re-assembly of components lost during gradient isolation. Our use of the *prp18* mutation affecting the second step of the splicing reactions enabled the development of conditions for the inactivation of the second reaction. Chasing experiments with *prp18* heat inactivated spliceosomes have defined a need for ATP and the dispensablity of intact snRNPs for complementation.

Molecular analysis of PRP18

The temperature sensitivity *prp18* mutation has allowed the cloning of the wild type gene and these experiments would be essential in the further analysis of the role of *PRP18* in splicing. The cloned gene in single or low copy vectors (*i.e.*, CEN vectors ; YCp50 and pPHY18) is capable of rescuing the temperature sensitive phenotype and the *in vivo* splicing defect . The cloned DNA has been characterized by restriction mapping and subcloning; a ~2.5 kb genomic fragment has been shown to complement both phenotypes. This fragment is unique to the yeast haploid genome. The cloned DNA has been shown to be genetically linked to the *PRP18* locus by integrative transformation, followed by segregation and southern analysis. These studies showed that the cloned DNA is the wild type *PRP18* locus and not a suppressor of the *ts* and splicing phenotype. Transformation of the cloned DNA on multicopy vectors does not alter the complementation of phenotypes, and hence over-expression of the protein-using yeast vectors would probably not be deleterious to the cell.

A combination of probes spanning ~2.5kb of complementing DNA fragment were used to identify the *PRP18* transcript. These experiments were necessary as the larger yeast genomic fragment, when used as probe, hybridized to more than one polyA$^+$ RNA. The *PRP18* gene product is most likely encoded by a poly-adenylated transcript of ~2.3kb. Further molecular characterization of the locus, including sequencing of the *PRP18* gene and over-production of the protein for the generation of antibodies are experiments in

progress, and these probes should enable further analysis of this newly identified splicing factor.

MATERIALS AND METHODS

Strains and microbiological techniques

The yeast strains used in this study are summarized as : wild type EJ101 and SS330, the *prp5* strain was RL172 and the *prp11* strain was SPJ11.4, the genotypes of these strains are as described in Lustig *et al.* (1986) and Vijayraghavan, Company and Abelson (manuscript submitted) . The *prp18-2* strains were obtained after outcrossing *ts* 503 with wild type strain SS330, and were 503-1a *MATa , ade2-101, his3 Δ 200, ura3-52, lys 2-801* and 503-6c *MATa, ade2-101, his3Δ200, ura3-52 , lys2-801*. The yeast strains were propagated by standard methods on media as described by Sherman *et al.* (1986). Temperature sensitive strains were maintained at 23°C, and 37°C was used as the non-permissive temperature. Sporulation, omission and rich media were prepared as in Sherman *et al.* (1986). Yeast transformations were all done by the lithium acetate procedure of Ito *et al.* (1983). Transformants were selected on omission media at 23°C and colonies were then replica plated to pre-warmed rich plates (YPD) at 37°C to test for temperature resistance. The YCp50 based Sau3A library of *S. cerevisiae* genomic DNA was obtained from M. Rose and P. Novick. The yeast shuttle vector pPHY18 (CEN, ARS, URA3 Amp^r) and pSEY18 (2m, ARS, URA3, Amp^r) used in sub-cloning experiments was kindly provided by P. Herman and S. Emr. Selection for the loss of yeast CEN, URA3 plasmids was done on 5- fluoro-orotic acid plates prepared as described in Boeke *et al.* (1984). The *E. coli* strain used for cloning and DNA amplification was MC1061 [F^- *araD139 Δ (ara ABIOC-leu) 7679 Δ(lac) x74 galU galK rpsL hsr* hsm^+].

Nucleic acid manipulations

Restriction mapping and standard cloning techniques were essentially as in Maniatis *et al.* (1982). Subcloning of restriction fragments from the plasmids was done after isolation of

the desired restriction fragment, and of the suitably digested vectors : pPHY18, pSEY18 or YIP5, from low melting agarose gels. The plasmids from *E. coli* cells were analyzed after alkali lysis preparation of DNA. Probes, when used, were restriction fragments obtained from low melting agarose gels; and were generated with the random primer labeling procedure of Feinberg and Vogelstein (1983). Small scale preparations of yeast genomic DNA were done as described in Sherman *et al.* (1986). Southern blots were performed as suggested by New England Nuclear. Hybridizations were conducted in conditions similar to that in Cheng and Abelson (1987).

Preparation of total RNA from yeast was done as described in Vijayraghavan, Company and Abelson (manuscript submitted) and polyadenylated RNA was purified by oligo-dT cellulose affinity chromatography essentially as given in Maniatis *et al.* (1982). The RNA was separated on formaldehyde agarose gels before blotting on a nylon membrane. Primer extension on 20mg of total RNA, with an RP51 oligonucleotide hybridizing 70 nts downstream of the 5' splice site as a primer, was performed with reverse transcriptase as described in Vijayraghavan, Company and Abelson (manuscript submitted).

Preparation of splicing extracts

Extracts were isolated from cells at mid-to-late logarithmic phase grown in YPD at 23°C by the spheroplast procedure of Lin *et al.* (1985). The protein concentration of the extracts was approximately 20 mg/ml. Fractions 40P3 and 40W from extracts of wild type EJ101 cells were provided by Dr. S.-C. Cheng.

In vitro splicing reactions, heat inactivation and complementation

Sp-6 actin transcripts were generated using Sp-6 polymerase with UTP at 100μM and ^{32}P-UTP at 2mCi/ml. Heat inactivation of splicing extracts involved use of 7μl of extract to which was added 2.2μl of an inactivation cocktail such that inactivation conditions were at 2.4 mM $MgCl_2$, 1mM DTT and RNasin 12u. Inactivation was done at 30 to 32°C for varying periods and time points were taken and kept on ice until completion of the last time point, at which time splicing was initiated. Splicing cocktail was added to obtain a final

concentration of 2.2 mM ATP, 55mM KPO_4 and 2.7% PEG, with 5000-10000 cpm (~3.3fmol) of labeled actin pre-mRNA. Splicing was carried out at 15°C for 30 to 60 minutes and reaction mixture was phenol extracted and ethanol precipitated. The precipitated RNA was analyzed on denaturing polyacrylamide gels as described by Lin *et al.* (1985). Complementation *in vitro* was tested by mixing approximately equal amounts of individually inactivated extracts before initiation of splicing. Fractions of wild type extract were diluted in inactivation cocktail and were then added to inactivated *prp18* extracts.

Glycerol gradient sedimentation of splicing reactions

50µl splicing reactions were chilled on ice and diluted with cold gradient buffer (0.1M KCl, 2mM $MgCl_2$, 20mM HEPES pH7.4) such that the density of the sample was lower than that of the top of the gradient. The diluted sample was layered carefully on a 15-27% glycerol gradient made in 1/2 inch to 2 inch poly allomer tubes. The sedimentation was done at 50,000 rpm for about 100 min. at 2 to 4°C, in an SW55 rotor. Fractions were collected on ice and Cerenkov counts of the fractions determined. Fractions of interest were frozen in liquid N_2 and stored at -70°C. A small aliquot from each fraction across the 40S region of the gradient was phenol extracted and ethanol precipitated. The samples were analyzed on non-denaturing gels, to confirm that the 40S region contained the expected labeled RNA molecules.

Chasing reaction of gradient isolated spliceosomes

Conversion of the lariat intermediate and exon1 to products- lariat intron and mRNA was termed a chasing reaction. Typically 20µl reactions with 14µl of the appropriate gradient fraction, 1µl of 10x chasing buffer (200 mM KPO_4 pH 7, 11mM $MgCl_2$, 20mM ATP 14% PEG 8000) and 2µl of complementing extract was incubated at 15°C for 60 minutes. Fractions of wild type extract when used for complementation were added at half the volume of other complementing extracts. The reaction products were recovered by phenol-chloroform extraction and were separated on polyacrylamide gels.

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FIGURE LEGENDS

Fig 1A. Splicing activity in *prp18* and wild type extracts

Splicing assays were conducted at 15°C for 30 or 60 min. as indicated in the figure. Extracts from two different *prp18* strains, *ts* 503-1a and *ts* 503-6c, and from the wild type strain SS330 were assayed. These extracts were used, without any heat inactivation, in splicing conditions described in Materials and Methods. The migration position of the lariat intermediate (IVS*E2), the lariat intron (IVS*), spliced exons (mRNA) and the exon1 (E1) are indicated in the figure.

Fig. 1B. Heat inactivation of splicing extracts from *prp18*

ts 503-6c extracts were inactivated in conditions described in Materials and Methods for increasing periods of time (0 to 60 min.) at 32°C. After inactivation, splicing is initiated with the addition of labeled actin pre-mRNA in a cocktail, and splicing is conducted at 15°C for 60 min. Reaction products are fractionated on denaturing acrylamide gels. The RNA species labeled IVS*E2, IVS*, pre-mRNA, mRNA and E1 are the same as explained in panel A.

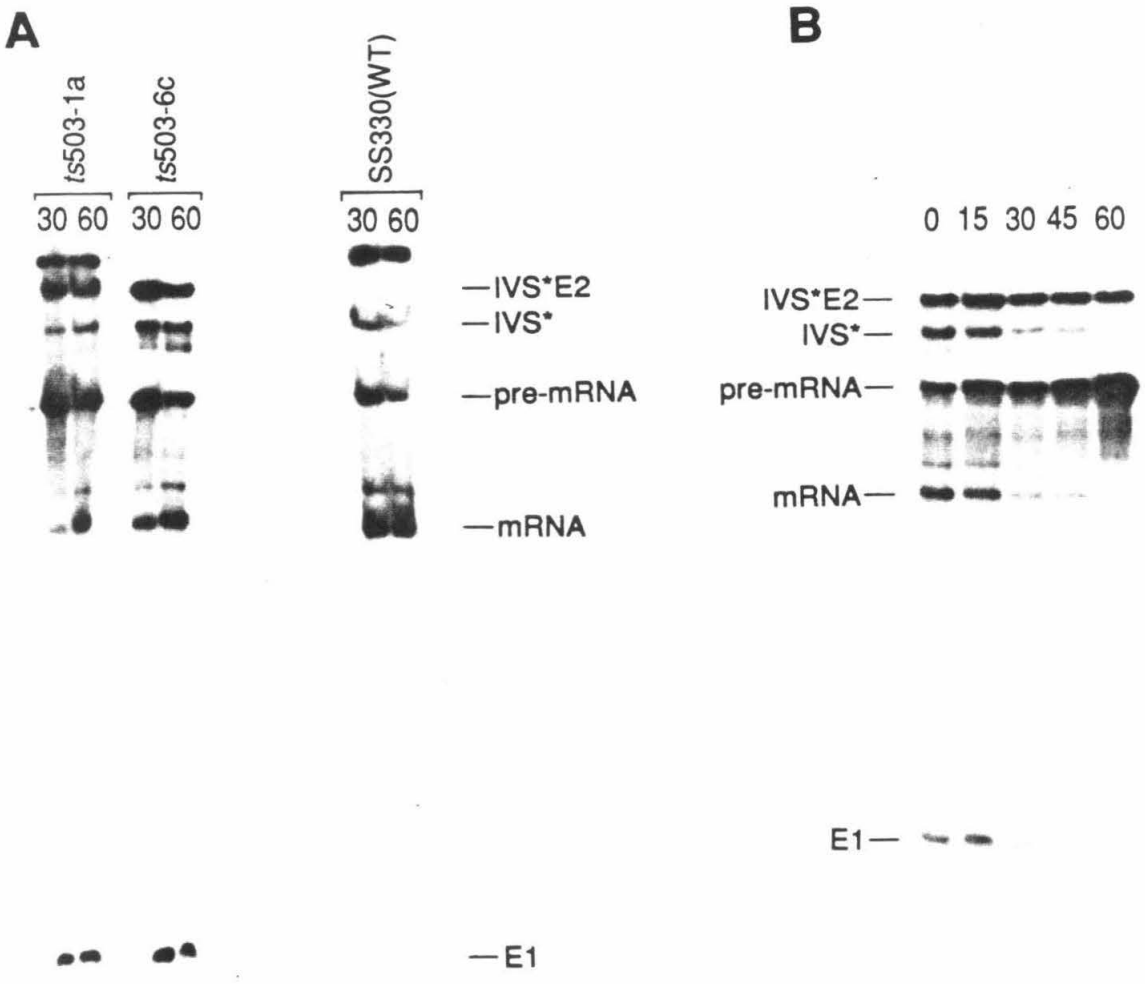

A
ts503-1a
ts503-6c
SS330(WT)
30 60
30 60
30 60
—IVS*E2
—IVS*
—pre-mRNA
—mRNA
—E1
B
0 15 30 45 60
IVS*E2—
IVS*—
pre-mRNA—
mRNA—
E1—

Fig 2. Complementation of heat inactivated *prp18* extract.

prp18 (*ts* 503-6c) extract, inactivated at 32°C for 70 min., was assayed individually (lane7), and in combination with either heat inactivated (D) *prp5* extract (lane 1), or *prp11* (lane 2) or in combination with a different inactivated *prp18* (*ts* 503-1a) extract (lane 6). Combinations of *prp18* inactivated extracts and fractions of wild type extract 40P3 (lane 3) or 40W (lane 4), or micrococcal nuclease-treated wild type extract (lane 5) were also assayed for complementation. After mixing of extracts, the splicing reactions are initiated with the addition of splicing cocktail and incubation at 15°C for 60 min. The reaction intermediates and products are indicated and described in Fig. 1A. The delta sign (Δ) signifies heat inactivated extracts.

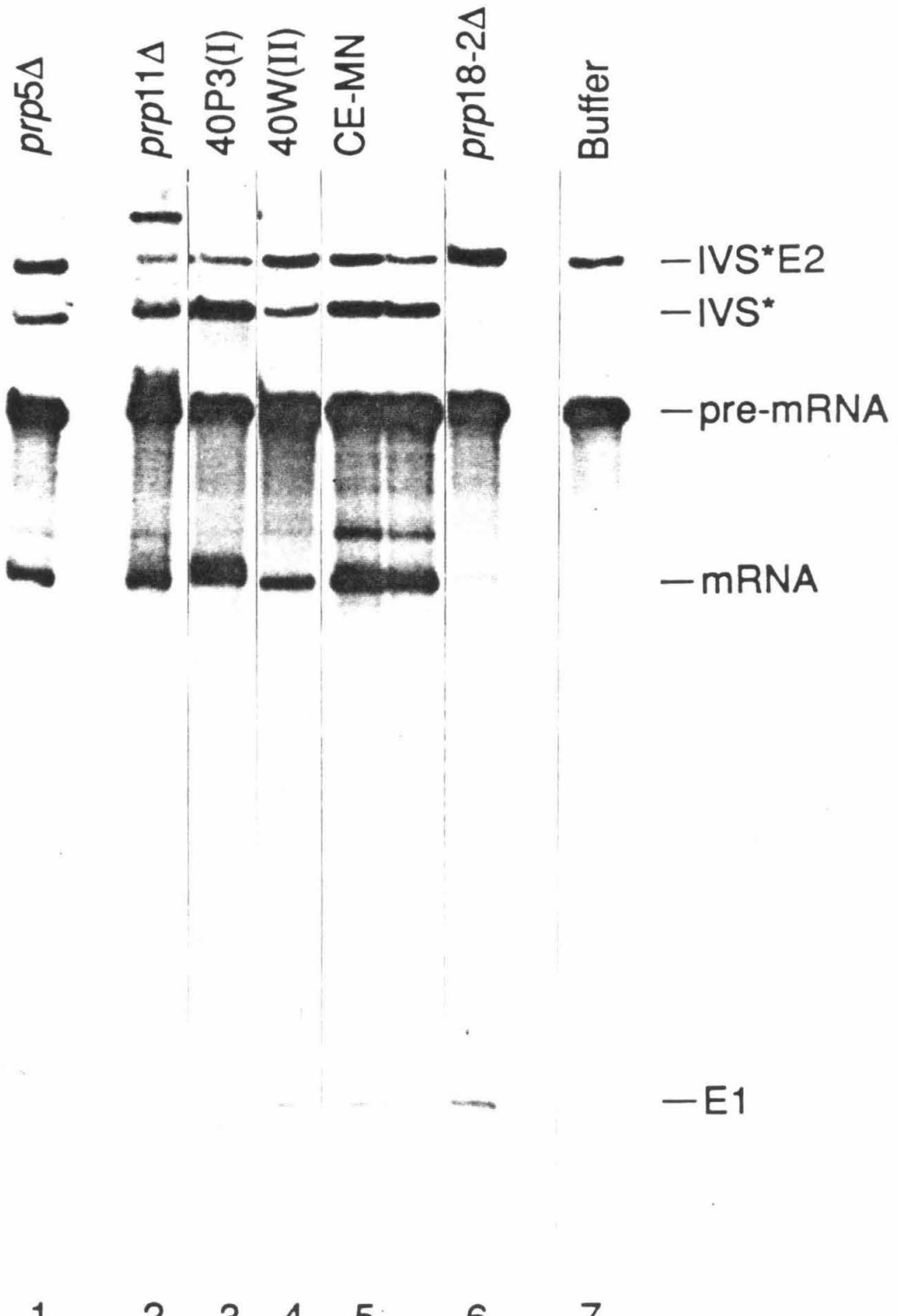

prp5Δ
prp11Δ
40P3(I)
40W(II)
CE-MN
prp18-2Δ
Buffer
—IVS*E2
—IVS*
—pre-mRNA
—mRNA
—E1
1
2
3
4
5
6
7

Fig 3A. Chasing of heat inactivated (Δ) *prp18* spliceosomes

The gradient profile of a 50ml splicing reaction conducted with inactivated *prp18* extracts. The reaction mixture was sedimented for 100 min. and analyzed as described in Materials and Methods. Analysis of the labeled RNA in an aliquot of the gradient fractions (data not shown) revealed a peak of lariat intermediate at fraction 12, corresponding to the peak of ^{32}P labeled RNA in the 40S region of the gradient.

Fig 3B. Complementation of *prp18* (D) spliceosomes. Fraction 12 from the gradient described in panel A, was used as the *prp18* heat inactivated spliceosome. The chasing of the intermediates in the spliceosome was done as described in Materials and Methods. No complementing extract was added in lane 7, lane 1 was incubated with inactivated *prp11* , lane 2 with inactivated *prp5*, lane 3 with fraction 40P3 from wild type extract, lane 4 with micrococcal nuclease-treated 40P3, lane 5 with 40W fraction of wild type extract, lane 8 was incubated with inactivated *prp11* extract in the absence of ATP in the chasing mix. The products of the chasing reaction, lariat intron (IVS*) and the spliced exons (mRNA), are indicated in the figure.

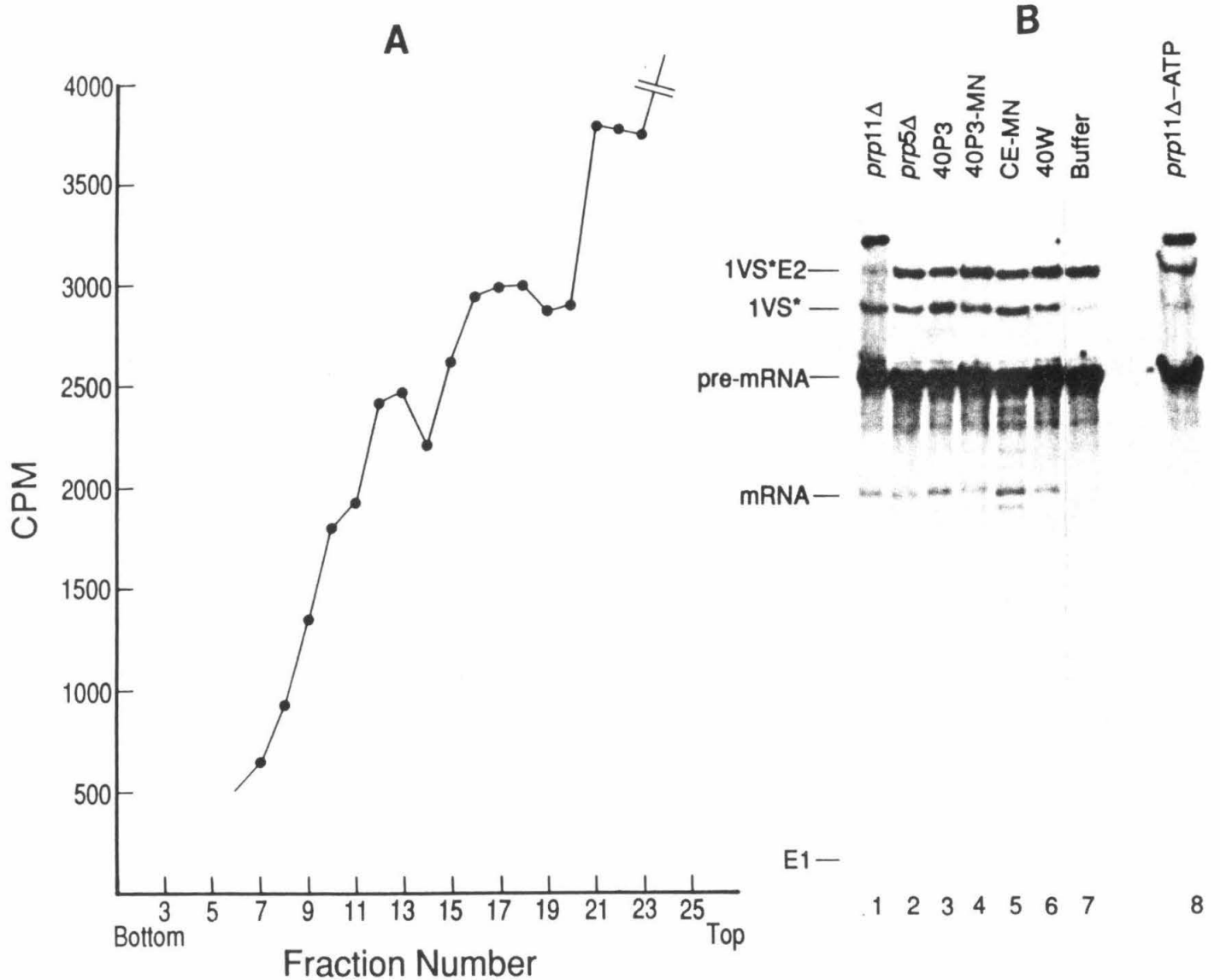

A
CPM
4000
3500
3000
2500
2000
1500
1000
500
3 5 7 9 11 13 15 17 19 21 23 25
Bottom
Top
Fraction Number
B
prp11Δ
prp5Δ
40P3
40P3-MN
CE-MN
40W
Buffer
prp11Δ–ATP
1VS*E2
1VS*
pre-mRNA
mRNA
E1
1 2 3 4 5 6 7 8

Fig 4. In vivo complementation analysis with clones and subclones of *PRP18* .
Restriction map of pUV18 and complementation phenotype for the subclones with portions of the genomic DNA fragment in pUV18. Only restriction sites relevant to the isolation of subclones and the generation of hybridization probes are indicated. + indicates ability to suppress the temperature sensitive growth defect of *prp18* , and - incidates inability to confer temperature insensitivity. The wavy line represents YCp50 sequences and the hatched box the complementing region. Restriction enzymes : B, *Bam* HI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; N, *Nru* I, S, *Sal* I and X, *Xho* I.

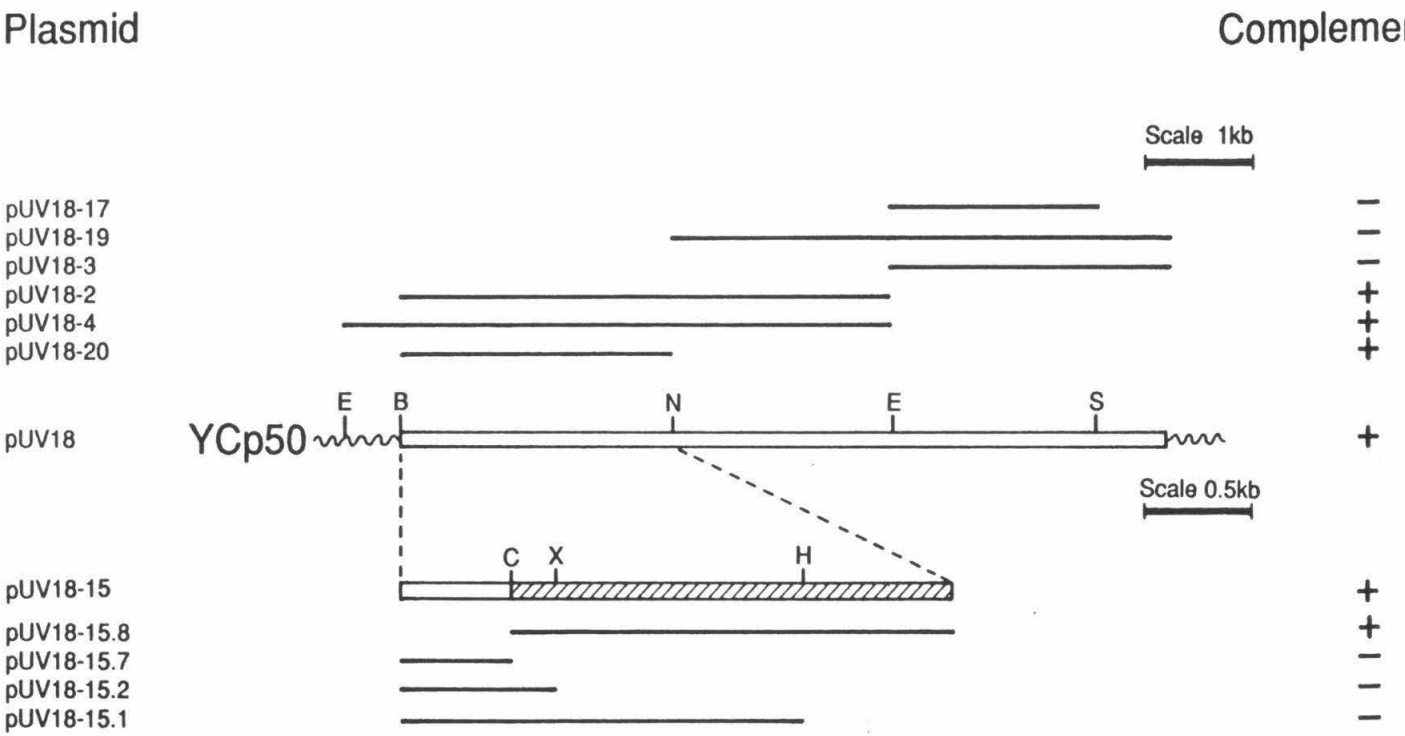

Plasmid
Complementation
Scale 1kb
pUV18-17
pUV18-19
pUV18-3
pUV18-2
pUV18-4
pUV18-20
E
B
N
E
S
pUV18
YCp50
Scale 0.5kb
C
X
H
pUV18-15
pUV18-15.8
pUV18-15.7
pUV18-15.2
pUV18-15.1
− − − + + + + + − − −

Fig 5A. Complementation of lariat intermediate accumulation phenotype of *prp18*

Total RNA (10mg in each case) prepared from cultures at 23°C and 37°C was electrophoresed through a 1.2% formaldehyde agarose gel, blotted and crosslinked to GeneScreen. The blot was then probed with a labeled fragment of the actin intron. Lanes 1 and 2 were loaded with RNA from a *prp18* strain transformed with the vector YCp50, lanes 3 and 4 with RNA from *prp18* transformed with the complementing subclone pUV18, lanes 5 and 6 *prp18* transformed with complementing subclone pUV18-15 and lanes 7 and 8 with RNA from the untransformed *prp18* strain. The position of the actin lariat intermediate is indicated.

Fig 5B. Suppression of lariat intermediate phenotype of RP51A

Reverse transcription was done to map the 5' end of RP51A transcripts. 20mg of total RNA was hybridized to a RP51A oligonucleotide complementary to sequences 70nts downstream from the 5' splice site. The extension products are separated on 10% 8M urea acrylamide gels. RNA from untransformed wild type and *prp18* strains, the *prp18* strain transformed with the complementing plasmid pUV18, *prp18* strain transformed with the subclone pUV18-15, *prp18* strain transformed with a complementing multicopy plasmid were used. The last lane contains kinased oligonucleotide markers of sizes 70, 55 and 35 nts. The strong extension stop seen in *ts* 503 just above the 70 nts marker corresponds to a reverse transcription stop at the 5' end of the accumulated lariat intermediate in the mutant strain.

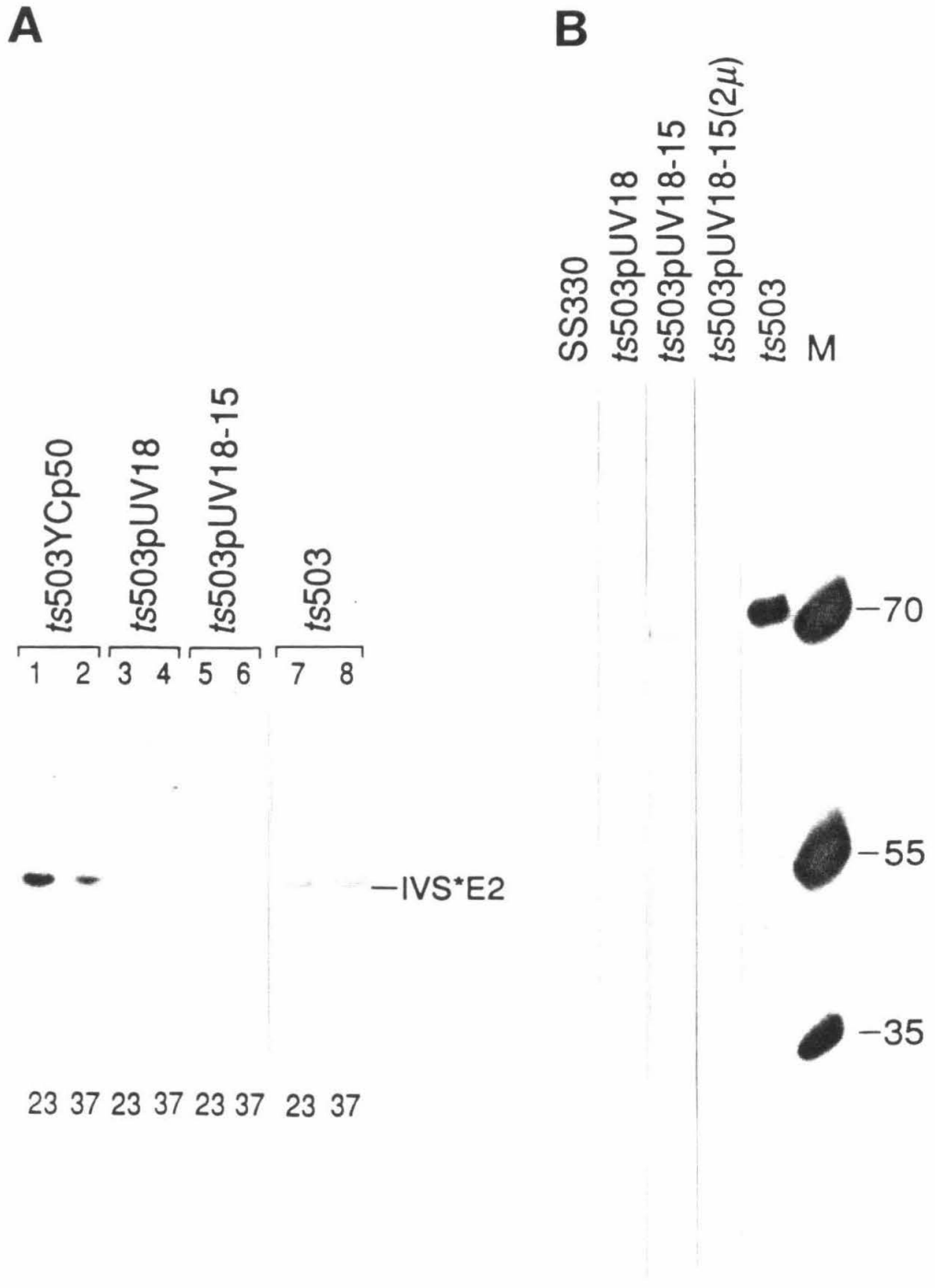
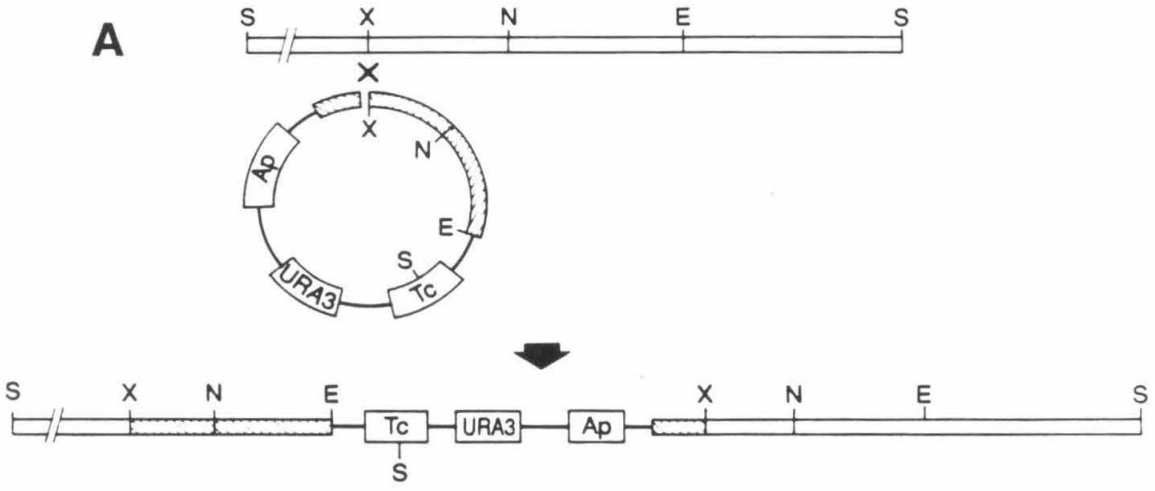

A
ts503YCp50
ts503pUV18
ts503pUV18-15
ts503
1 2 3 4 5 6 7 8
—IVS*E2
23 37 23 37 23 37 23 37
B
SS330
ts503pUV18
ts503pUV18-15
ts503pUV18-15(2μ)
ts503
M
—70
—55
—35

Fig 6A. Integration of the cloned DNA to the homologous choromosomal *PRP18* locus Cartoon of the expected integration event. A 5 kb complementing *Eco* RI fragment (see Fig 4) subcloned in YIP5 was linearized at the unique *Xho* I site and transformed in the wild type strain SS330. The open box represents the chromosomal locus and the cloned yeast DNA is represented by the hatched box. Plasmid sequences are represented by thin solid lines and markers in the plasmid as labeled boxes (Ap represents ampicillin resistance, Tc represents tetracycline resistance and URA3 is the yeast marker gene). Restriction sites are represented by the same notations as in Fig. 4.

Fig 6B. Southern blot confirming integration event at the cloned genomic locus. DNA from wild type, *prp18* strain and from two integrants SS330 Ia and Ib was digested with *Sal* I and run on a 1.0% agarose gel, blotted and probed with the 4.5 kb *Bam*HI to *Eco*RI (see Fig. 4) complementing fragment.


A
S
X
N
E
S
X
Ap
N
E
S
URA3
Tc
S
X
N
E
Tc
URA3
Ap
X
N
E
S
S

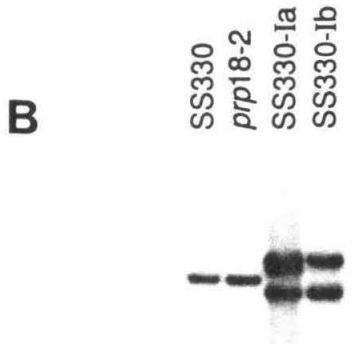
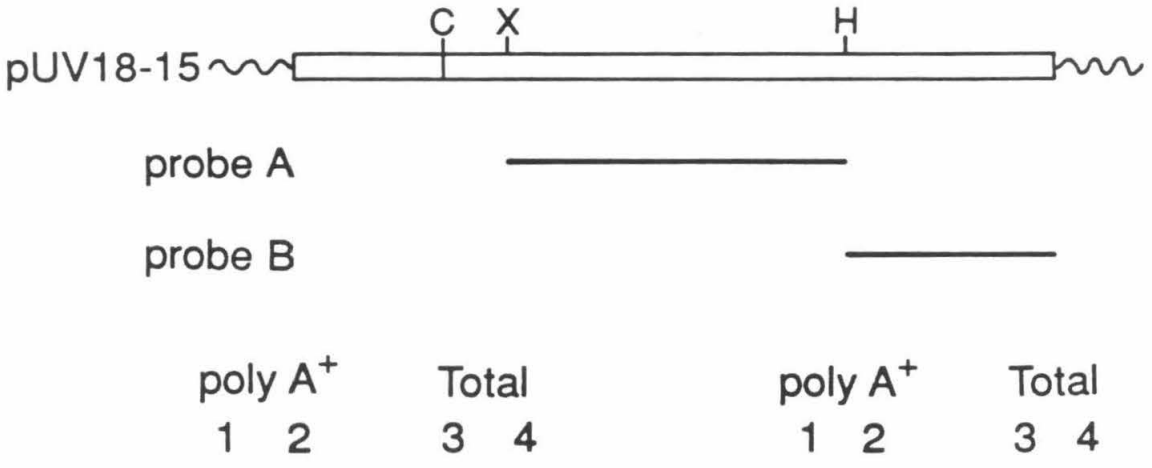
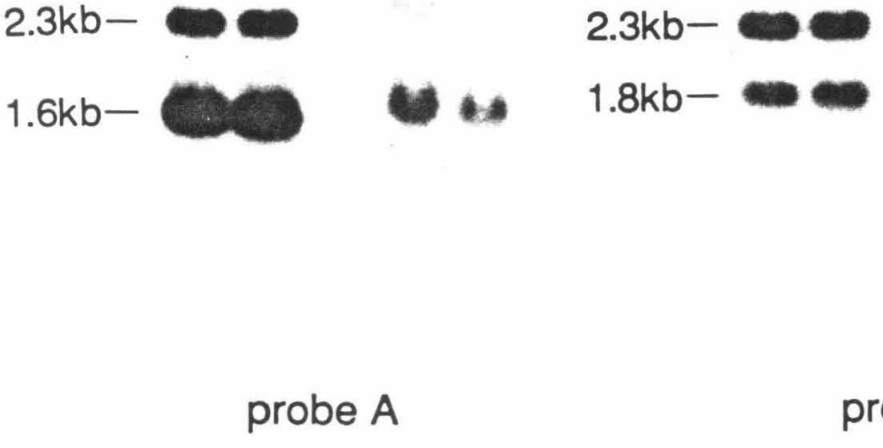

B
SS330
prp18-2
SS330-Ia
SS330-Ib

Fig 7. Mapping the *PRP18* transcript.

A restriction map of the complementing fragment containing the *PRP18* gene is represented by the open box, and the wavy lines represent vector sequences. Probe A and Probe B were used on a northern blot having 2mg of poly A^+ RNA (lane 1 and 2); and 10 and 20 mg of total RNA (lanes 3 and 4) prepared from wild type cells. The sizes of the transcripts were determined by probing the blot for known transcripts (*STE2* and *TCM1*) and also by running DNA markers (l *Hin*dIII) on the gel.


C
X
H
pUV18-15
probe A
probe B
poly A+
Total
poly A+
Total
1 2
3 4
1 2
3 4


2.3kb—
1.6kb—
2.3kb—
1.8kb—
probe A
probe B

CHAPTER 4

Intron stabilization in the yeast mutant *prp26*

ABSTRACT

We have recently reported the isolation of a novel splicing mutant *prp26* whose phenotype is intron accumulation. Further analysis of this mutant phenotype reveals that the intracellular intron exists in a lariat and a circular form lacking nucleotides from the 3' end of the lariat. The stabilization of the cellular intron results from its association with a large particle having sedimentation properties similar to that of the spliceosome (~40S). The composition of this intron-containing particle was analyzed after gradient sedimentation by northern analysis for the presence of the intron and snRNAs. A co-sedimentation of spliceosomal snRNAs and the intron was observed. Antibodies that precipitate snRNPs: tri-methyl G cap and anti-Sm antibodies, immunoprecipitate the intron. Splicing extracts prepared from strains bearing this mutation are active for splicing but are defective *in vitro* in the degradation of *in vitro* generated intron.

INTRODUCTION

The removal of intervening sequences from the primary transcript occurs by RNA splicing. The study of pre-mRNA splicing in *in vitro* systems has facilitated the understanding of the mechanism of pre-mRNA splicing. A two step mechanism of splicing has been established (reviewed in Padgett *et al.*, 1986; Green 1986). In the first step cleavage at the 5' splice junction occurs with concomitant formation of splicing intermediates- a lariat RNA of intron-exon2 and exon1. The second reaction involves cleavage at the 3' splice site and ligation of the exons occurs. This cleavage ligation reaction releases the intron in a lariat form. Although, mechanistically, the splicing scheme is similar to that of the self-splicing GroupII introns (Cech 1986), nuclear pre-mRNA splicing depends upon the assembly and function of an RNA and protein complex termed the spliceosome (Brody and Abelson 1985; Grabowski *et al.*, 1985; Frendewey and Keller 1985; Perkins *et al.*, 1986; Bindereif and Green 1986).

Splicing complexes were first detected upon sedimentation analysis of splicing reactions as 40-60S particles. Small nuclear ribonucleoprotein particles (snRNPs) have been shown to be essential components for spliceosome formation and function (Steitz *et al.*, 1987; Guthrie and Patterson 1988). Purification of gradient isolated spliceosomes by affinity chromatography revealed the presence of U2, U4, U5 and U6 snRNAs (Grabowski and Sharp 1986). Identification of assembly intermediates of the spliceosome initially by sedimentation analysis and subsequently by non-denaturing gel electrophoresis has facilitated the development of an assembly pathway. An ATP-dependent binding of U2 snRNP is followed by the binding of U4, U5 and U6 snRNPs (Konarska and Sharp 1986; Pikielny and Rosbash 1986). Prior to the first splicing reaction U4 leaves the complexes (Cheng and Abelson 1987; Lamond *et al.*, 1988). Hence the splicing intermediates are found in a complex of U2, U5 and U6. Although the U1 snRNP is known to be essential for pre-mRNA splicing, it was not detected on these assembly intermediates. Analysis of the kinetic order of binding of snRNPs to the pre-mRNA by affinity chromatography has implied an early and ATP independent binding of U1 to the pre-mRNA, which is followed by the binding of U2, U4, U5 and U6 snRNPs (Bindereif and Green 1987; Ruby and Abelson 1988).

The absence *in vivo* of branched RNA in the cytoplasm and low levels of branched RNAs detected in the nucleus imply a rapid degradation of the lariat intron (Wallace and Edmonds 1983). A specific enzymatic activity that hydrolyzes 2'-5' phosphodiester bonds has been characterized in HeLa cell extracts (Ruskin and Green 1985; Arenas and Hurwitz 1986). However, *in vitro* some of the lariat intron is associated with snRNPs in large complexes similar to the spliceosome. These complexes are thought to be involved in the dis-assembly of the spliceosome (Konarska and Sharp 1987; Lamond *et al.*, 1987). The release of the intron as a particle explains the protection of *in vitro* generated intron from the effective debranchase present in HeLa cell extracts. The mRNA is released from spliceosomes and appears to be associated with hnRNP type complexes.

Analysis of splicing factors in yeast has the added advantage of the availability of temperature sensitive mutations (*prp 2-11*), which define protein components of the splicing machinery (reviewed in Warner 1987; Vijayraghavan and Abelson 1988). *In vitro* heat inactivation of extracts from these strains has implied a direct role for seven of the above complementation groups in the pre-mRNA splicing (Lustig *et al.*, 1986). All but one of these complementation groups (*prp2*) defines components required early in spliceosome assembly (Lin *et al.*, 1987). A recent search of more such mutants has obtained new complementation groups with novel phenotypes affecting late events in splicing. Two groups, *prp26* and *prp27* , define gene products required after splicing is completed, as accumulation of only the excised intron is observed (Vijayraghavan *et al.*, manuscript submitted). In this paper we have analyzed the biochemical effects of *prp26,* which causes accumulation of high levels of intron. We provide evidence for the stabilization of the intron in a large snRNP-containing complex, probably similar to the post splicing complexes observed in higher eukaryotic splicing systems.

RESULTS AND DISCUSSION

Accumulation of the branched intron

A novel yeast mutant with a phenotype of excised intron accumulation (*prp26*) was isolated upon screening for mutants in pre-mRNA splicing (Vijayraghavan *et al.*, manuscript submitted). This phenotype results from a trans-acting mutation in a single recessive gene. Primer extension analysis on the intron had indicated that the precision of 5' cleavage and branch site selection were unaffected. However, much less extension product was made from a primer hybridizing between the branch and the 5' splice site, than from the primer hybridizing downstream of the branch point. This suggested to us that some of the excised intron lacks nucleotides 3' to the branch site. In wild type cells no branched intron can be detected for at least four yeast transcripts tested. Only upon *in vivo* overproduction of an actin pre-mRNA fragment containing the intron, is the excised actin intron is detectable

(Domdey *et al.*, 1984). The excised intron consisted of four distinct forms- two of branched molecules and two linear forms of the intron.

To further characterize further the mutant phenotype in *prp26* , we analyzed the intron after fractionation of total RNA from *prp26* on 5% acrylamide, 8M urea gels to separate different forms of the intron. After electroblotting of the RNA, the membranes were probed with actin intron. They revealed the presence of three different forms of the actin intron in this mutant strain (Fig 1B). The largest form of the intron migrates at a position similar to that of the complete lariat form of the intron. The most abundant form of the intron has the same mobility as the circular form of the intron, which has lost some nucleotides 3' to the branch. A minor species of the intron, corresponding to a linear form, again lacking the 3' nucleotides, is also detected. These RNAs were assigned on the basis of their mobility and by comparison with published results (Domdey *et al.*, 1984). None of these species is detectable in the wild type parental strain. That these forms of the intron are normal intermediates in the degradative process of the intron is indicated by the correlation between the forms of the intron seen in this mutant strain and those observed *in vivo* upon overproduction of actin intron containing fragment . The composition of the intracellular intron in *prp26* is thus predominantly branched, although the lariat intron itself is not the major form of the intron. The presence of the circular intron probably results from nucleolytic digestion of the nucleotides 3' to the branch site. The small amount of the linear intron observed suggests that the normal degradative pathway functions to some extent in *prp26* .

Association of the intron in a 40S particle

The stabilization of the branched intron in the cell where it is normally degraded, suggests that it exists in an intracellular complex. One interpretation of the presence of the branched RNAs is that the enzymatic debranching function, responsible for degradation of the unique 2'-5' linkage in these branched molecules (Ruskin and Green 1985; Arenas and Hurwitz

1987), is defective in the mutant strain. Alternatively, protection of the released intron because of a defect in the dis-assembly of the splicing components may be responsible for branched intron accumulation. We therefore investigated the possibility that this mutation *prp26* results in a protection of the intron due to its association with spliceosomal components.

Whole cell extracts prepared from *prp26* were fractionated on glycerol gradients. In parallel, deproteinized RNA from the same extract was also sedimented, to provide a control for the migration of the naked RNA. Another gradient of an *in vitro* splicing reaction with labeled pre-mRNA provided a marker for the position of the 40S spliceosome. The gradient fractions obtained from the mutant extract or the naked RNA were individually extracted and their RNA content analyzed on northern blots. These blots were probed with a labeled fragment of the yeast actin intron. A peak of the intracellular lariat intron, the circular intron and the minor amount of the linear intron co-sedimented in the 40S region of the gradient (Fig. 1A). In fact this intron containing particle sedimented nearly as fast as the spliceosome (data not shown). The presence of all forms of the intron in the same region of the gradient suggested that they may exist in the same complex. In a parallel gradient of the naked RNA the lariat and circular RNAs sedimented at the top of the gradient (Fig. 1B). These results show the intron is contained in a fast sedimenting particle of 38-40S; the sedimentation coefficient suggests that the intron is retained in a large post-splicing particle.

Sedimentation of *in vitro* splicing reactions on gradients has demonstrated a co-sedimentation of the splicing intermediates in 40S spliceosome (Brody and Abelson 1985; Grabowski *et al.*, 1985; Frendewey and Keller 1985; Bindereif and Green 1986). The lariat intron is associated with both a large complex slightly lighter than the spliceosome (~40S) and in a heterogenous particle of about ~20S (Brody and Abelson 1985; Bindereif and Green 1986). The mRNA is released as a smaller, more heterogeneous particle of about 20S. Analysis of the assembly of higher eukaroytic spliceosomes on non-denaturing

gels has also revealed the presence of a large intron- containing complex that most likely represents a post-splicing complex (Konarska and Sharp 1987). After gradient isolation the large intron-containing complexes have been reported to be insensitive to *in vitro* enzymatic debranching (Bindereif and Green 1986).

The intron-containing complexes (~40S) that accumulate in *prp26* containing predominantly branched RNAs suggests increased stability of a post splicing complex. The stabilization is therefore unlikely to result from a defective debranchase. The detection of mostly branched intron in the mutant strain *prp26* , in large complexes, re-enforces the theory that the stabilization of the intron is due to association with spliceosomal snRNPs.

Intron-containing complex is associated with snRNPs

Co-sedimentation of snRNAs and intron complex

The blots of the gradient fractions used to detect the intron-containing particles were probed for the spliceosomal snRNAs- U1, U2, U4, U5 and U6 (Fig. 2A, B and C). U6 and U5 snRNAs co-sedimentation with the peak of intron is evident. The sedimentation of U4 with the intron is at about 38S. The larger yeast snRNAs U1 and U2 also appear to co-sediment. However, the analysis of the association of this intron complex with snRNAs through co-sedimentation analysis may be artifactual; because co-sedimentation does not indicate that they exist in the same particle. In fact, this analysis is influenced by the fact that endogenous multi-snRNP complexes have been detected in wild type cells, which behave as large complexes (Konarska and Sharp 1987; Cheng and Abelson 1987).

Immunoprecipitation of intron by anti-^{3}mG and anti-Sm antibodies

The association of snRNPs with the intron in *prp26* was tested with snRNA/P specific antibodies in immunoprecipitation experiments. One of the antibodies used is directed against the ^{3}mG cap of snRNAs and the other antibody tried was the human autoimmune serum, anti-Sm, which has been shown to cross react with, and thus precipitate, yeast snRNPs

(Riedel *et al.*, 1986; Siliciano *et al.*, 1987). The anti ^{3}mG or the control pre-immune serum bound to protein A-Sepharose beads were incubated with whole cell extracts prepared from the parent wild type strain or the mutant *prp26* strain. The bound RNAs were released by phenol extraction separated on denaturing gels and blotted to nylon membranes. The intron RNA was immunoprecipitated from *prp26* extract by both the anti-^{3}mG and anti-Sm sera (Fig. 3). As expected, because wild type extracts do not contain introns, none are immunoprecipitated. Antibodies directed against the *PRP4* gene product of yeast specifically precipitate U4 and U6 snRNA from yeast extracts (J. Banroques and J. Abelson, manuscript in preparation). These antibodies were also used to investigate if *PRP4* protein is associated with the intron particle, but no precipitation of the intron was observed (data not shown).

The other product of the splicing reactions, mRNA, is not associated with snRNAs and is thus not precipitated (Fig. 3) (Bindereif and Green 1986; Konarska and Sharp 1987; Lamond *et al.*, 1987). The absence of the mRNA in the immunoprecipitates implies that the precipitation of the intron is probably not due to non-specific binding of RNA to the beads or the antibodies. The fact that the immunoprecipitation occurs by specific recognition of snRNA cap epitope is indicated by the effective precipitation of the snRNAs U5 and U4 (Fig. 3) and the U1 and U2 snRNA (data not shown). The anti-Sm antibody used in these experiments largely recognizes the yeast U2, and to some extent the U1, snRNA, but does not precipitate the U5 and U4 snRNAs (this study ; S.-C. Cheng and J. Abelson unpublished data). These experiments together indicated a direct precipitation of cellular intron in *prp26* , due to its association with snRNPs.

Immunoprecipitation of large intron-containing complexes from higher eukaryotic splicing reactions indicates the presence of Sm determinants in these complexes, while the smaller intron complexes are not precipitable (Bindereif and Green 1986). The association of U5, U6 and U2 with the excised intron of higher eukaroytic pre-mRNAs has been shown by gel electrophoresis (Konarska and Sharp 1987). Our finding that the endoge-

nous intron complex in *prp26* is Sm precipitable also supports the earlier suggestion that the Sm determinants protect the branch site during splicing reactions and after splicing they partition with the excised intron. These analyses imply that the stabilization of the intron in this mutant strain occurs through association in a large post-splicing complex. It is also possible that a defective debranchase is a part of this post-splicing complex and the dissociation of the complex can take place only after cleavage of the branch nucleotide. This is a possibility that has to be reconciled with the fact that the complex that accumulates in *prp26* seems similar to the *in vitro* generated intron complex observed in *in vitro* splicing with HeLa extracts, where this complex was insensitive to endogenous, and also to subsequently added, debranchase (Bindereif and Green 1986).

In vitro phenotype of intron accumulation

The stabilization of the cellular intron appeared to result from association with snRNPs. This observation suggested that splicing extracts prepared from the *prp26* mutant strain might be depleted for splicing activity because of sequestration of the snRNPs in the endogenous intron complex. The effect of this mutation on *in vitro* splicing was investigated. Splicing extracts were prepared from the original mutant isolated *prp26* (*ts 45*) and also from a strain obtained by twice outcrossing *prp26* with the wild type strain. Splicing assays with labeled actin pre-mRNA were conducted at either 15°C (permissive for mutant strains) or 23°C. The accumulation of intron was observed in extracts from both strains (Fig. 4). In extracts from wild type strains most of the generated intron is degraded, and the ratio of intron to mRNA is much less than one (Fig. 4). In the mutant extracts this ratio is nearly equal to one, due to the stabilization of the intron. The splicing *in vitro* must imply that the intron complex containing the cellular introns must be unstable with sufficient turnover for initiation of subsequent reactions. The same reason probably applies to the fact that this mutation is not lethal to the cell. The accumulation of intron *in vitro* provides a means for assay and purification of the wild type gene product through the use of a com-

plementation assay. Cloning of the wild type allele should be possible by assaying for the absence of introns in mutant strains transformed with a yeast genomic library.

MATERIALS AND METHODS

Strains : The yeast strains used in this report are the wild type strain SS330- *Mat a ade2-101 ura3-52 his3-Δ 200 tyr1* and the *prp26* strains *-ts* 45 *Mat α ade2-101 ura3-52 his 3Δ 200 lys 2-801* and the twice outcrossed strain 5a-3c- *Mat a ade2-101 ura3-52 his 3-Δ 200 tyr1* . The yeast strains were grown at 23C for splicing extract and RNA preparations.

Splicing extracts and in vitro splicing reactions : Extracts were prepared from all strains at mid-late logarithmic growth phase in YPD. The preparation of extract was done as detailed in Lin *et al.* (1985). Splicing reactions were performed in a buffer with 55 mM potassium phosphate (pH7), 2.5 mM $MgCl_2$, 2.4% PEG 8000, 2 mM ATP with 5000-10000 cpm of labeled actin pre-mRNA (~3.3 fmol) with 60% of the splicing extract. Splicing was carried out at 15C for 30-60 min. and at 23C for 10-20 min. The reaction mixture was extracted and analyzed on denaturing gels as described by Lin *et al.* (1985).

Gradient sedimentation of whole cell extracts : 25 µl of whole cell extract was diluted with 50µl of gradient buffer (0.1 M KCl, 2 mM $MgCl_2$, 20mM Hepes pH7.4). Total cellular RNA (~15 µg) when run on the gradient was also diluted in gradient buffer before layering on the gradient. 11-23% glycerol gradients were made in 1/2 inch to 2 inch polyallomer tubes. The sedimentation was done for 100 min. at 4C at 50,000 rpm. Fractions were collected on ice and the RNA was extracted with twice phenol-chloroform and ethanol precipitated. RNA from all fractions was loaded on 5% acrylamide (29:1), 8M urea gels. After electrophoresis, RNA from the gels was transferred to GeneScreen membranes (NEN) by electroblotting at 4C for 12-15 hrs at 30V in 25 mM sodium phosphate pH 6.5. The RNA was crosslinked as described in Cheng and Abelson (1987) and hybridized with the desired probes.

Northern blots : Prehybridizations and hybridizations were done similar to the procedure in Cheng and Abelson (1987). Probing for actin intron was done with a XhoI -ClaI fragment of the actin intron labeled by random primer extension (Feinberg and Vogelstein 1983). snRNA probes used for U1, U2, U4, U5 and U6 were prepared as described in Cheng and Abelson (1987).

Immunoprecipitation : Antiserum ^{3}mG, Sm or *PRP4* or the pre-immune serum when used were coupled to Protein A-sepharose in conditions as given in Cheng and Abelson (1986). 10µl of either wild type SS330 extract or mutant *prp26* extract was added to the beads. After incubation at 4C the beads were washed and extracted with phenol-chloroform and the RNAs precipitated. The RNAs were then fractionated on 5%, 8M urea gels and electroblotted on membranes, the RNA crosslinked and probed for actin transcripts and snRNAs.

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FIGURE LEGENDS

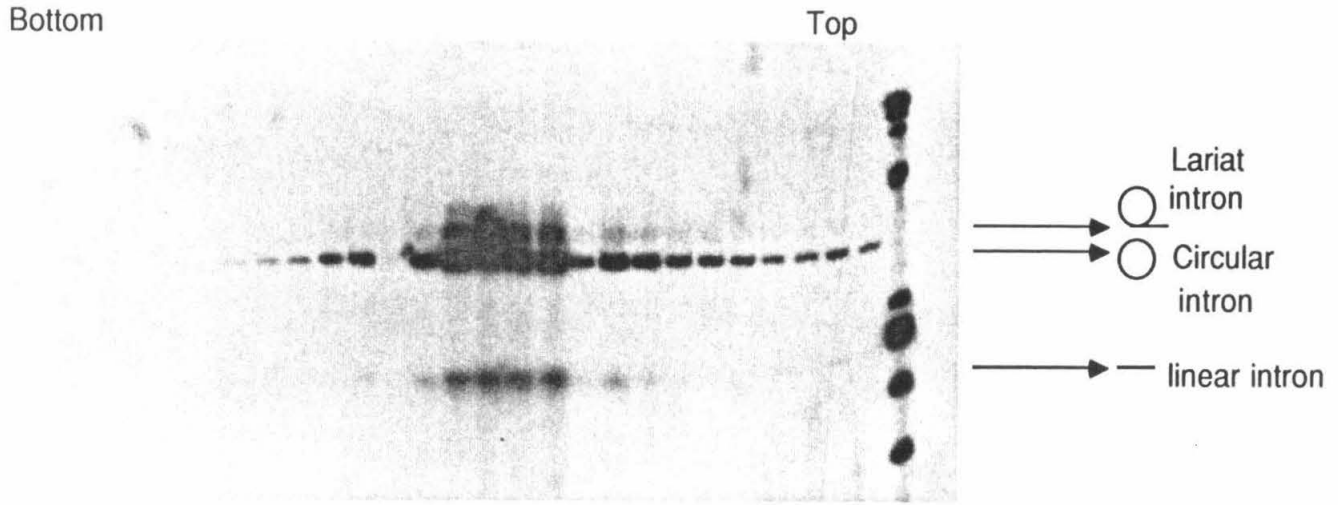
Fi. 1 Association of cellular excised intron with ~40S complexes

Fig 1A. Gradient sedimentation of whole cell extracts from *prp26*

Fig 1B. Gradient sedimentation of deproteinized RNA from *prp26*

Individual fractions from both gradients were collected on ice, and the RNA was extracted and fractionated on denaturing acrylamide gels. Electroblotting was followed by probing both blots for actin intron as described in Materials and Methods. Arrows indicate the position and form of the three endogenous actin intron types detected. The blot in panel A was subsequently used in hybridizations to snRNA probes.

A


Bottom
Top
Lariat intron
Circular intron
linear intron

B

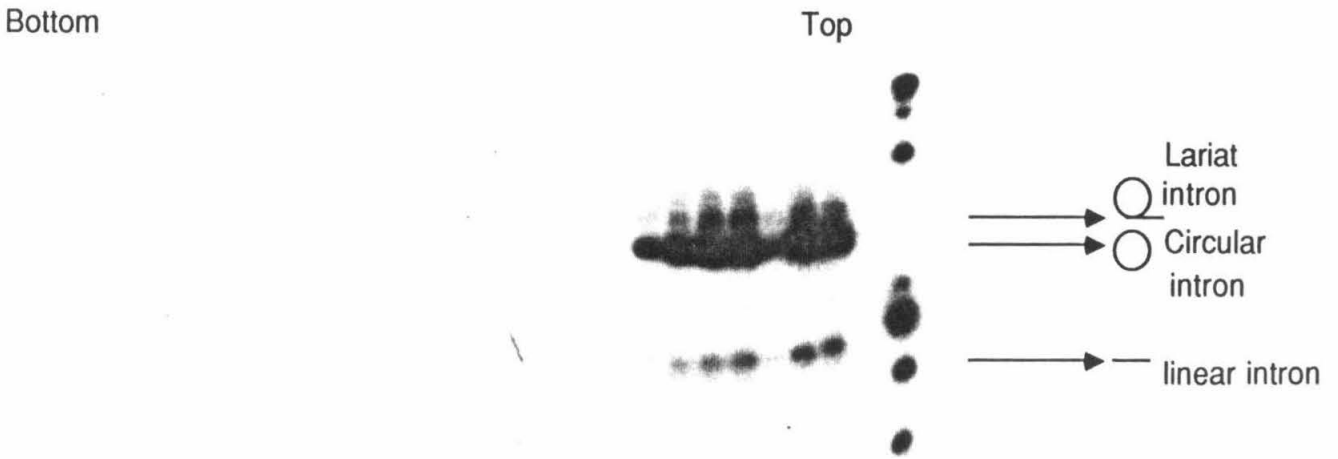

Bottom
Top
Lariat intron
Circular intron
linear intron

Fig 2 Analysis of snRNP sedimentation in extracts from *prp26*

Fig 2A. Reprobing of blot in Fig. 1 panel A, with yeast U1 and U2 snRNA probes. These probes were prepared and hybridized as described in the text.

Fig 2B. Detection of U5 and U4 particles in extracts of *prp26* after reprobing of blot in Fig1. panel A.

Fig 2C. Reprobing blot of intron-containing complex with a kinased oligonucleotide complementary to U6 snRNA detects U6 snRNP in *prp26* extracts.

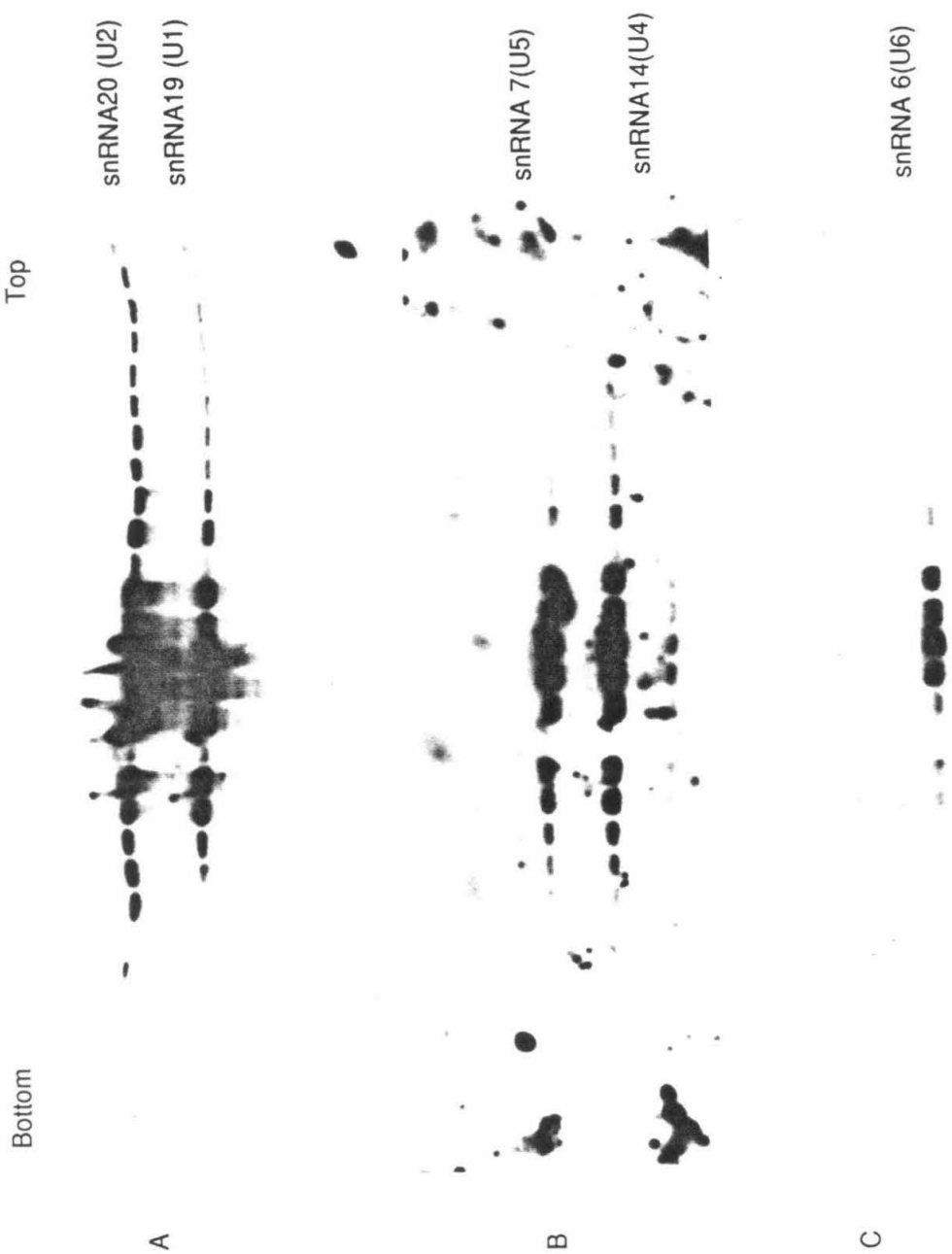
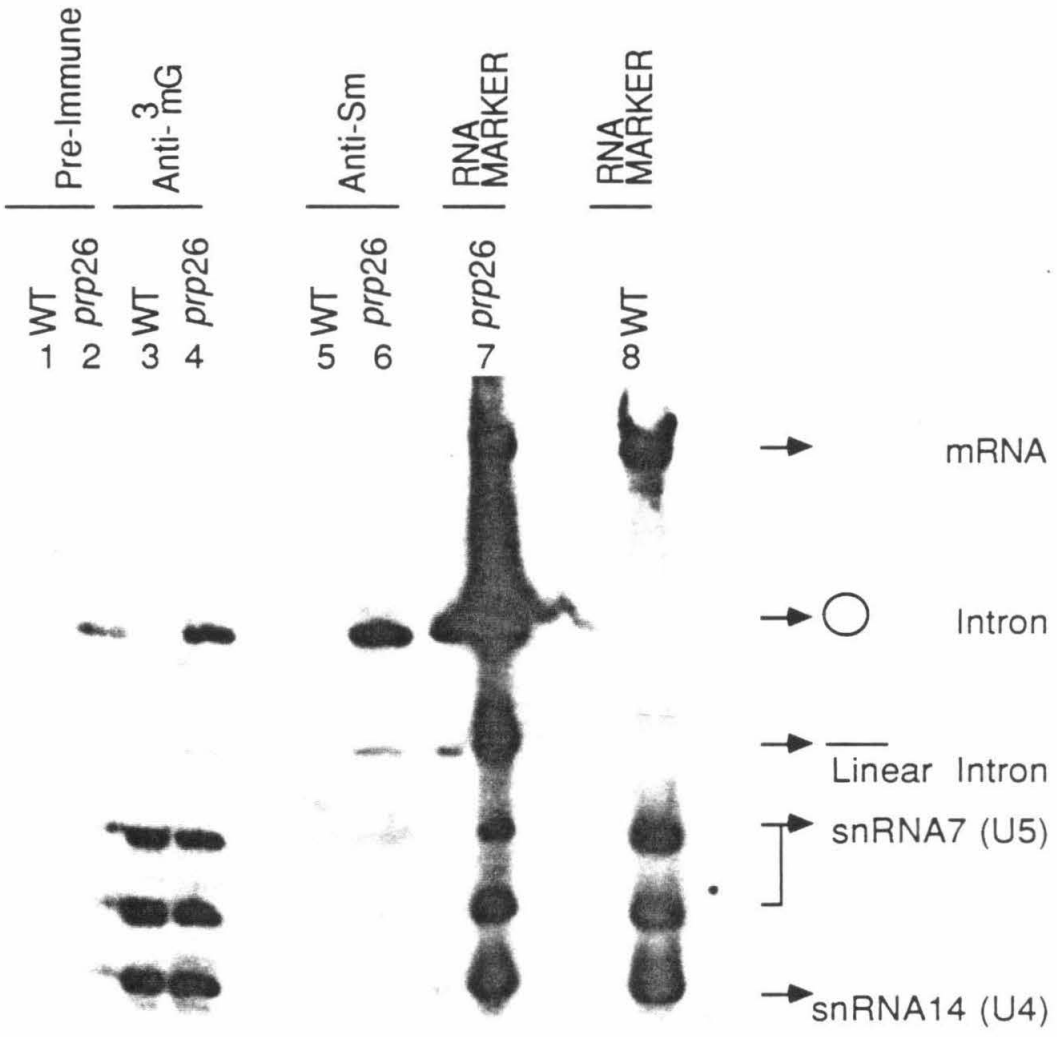

Top
Bottom
snRNA20 (U2)
snRNA19 (U1)
snRNA 7(U5)
snRNA14(U4)
snRNA 6(U6)
A
B
C

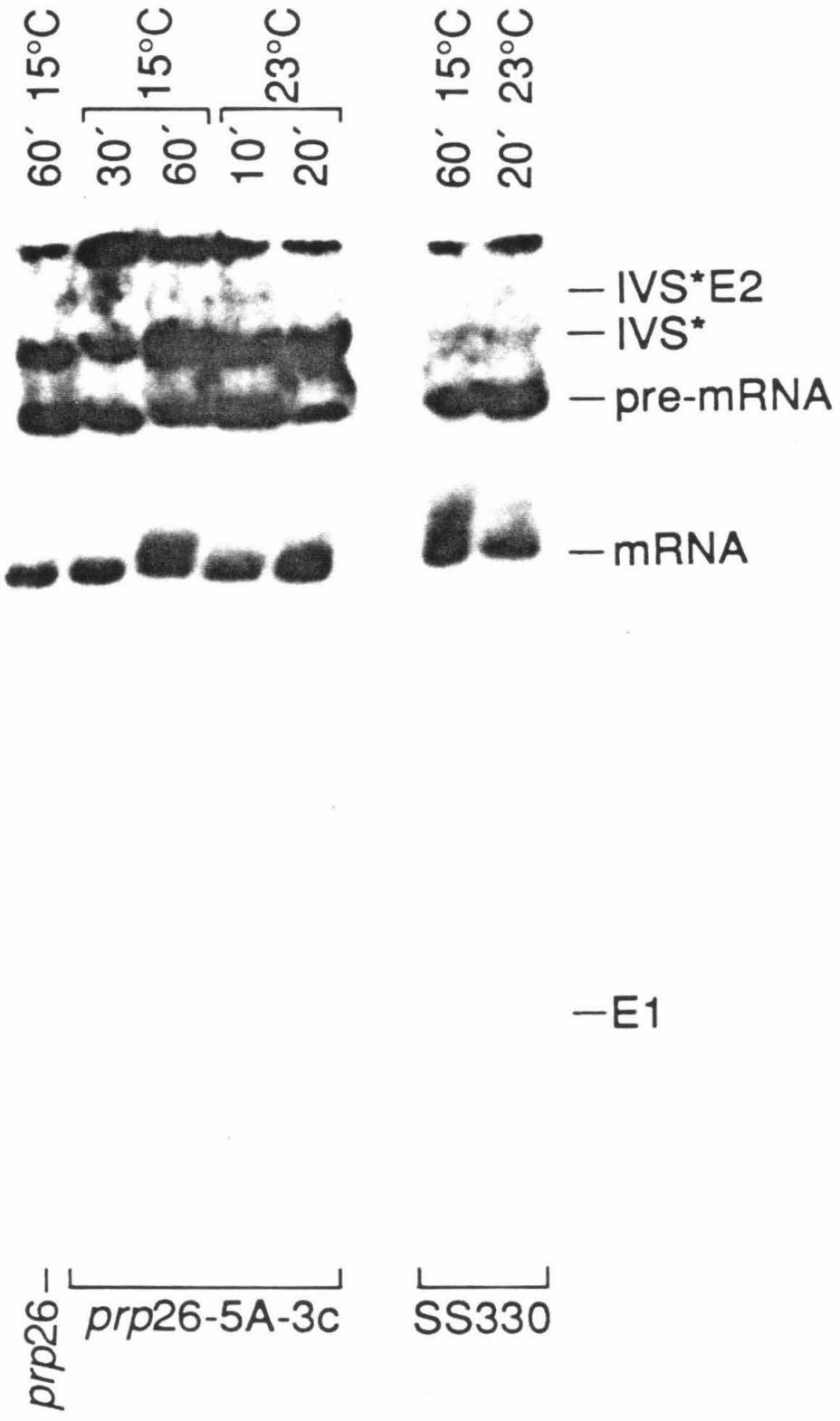
Fig 3. Immunoprecipitation of cellular intron from *prp26* with snRNA/P specific antibodies

Splicing extract from wild type or from the mutant *prp26* was incubated with preimmune (lanes 1 and 2), anti ^{3}mG(lanes 3 and 4) and anti-Sm (lanes 5 and 6) as indicated. RNA was extracted from the immune complexes and fractionated on denaturing gels and electroblotted. The blot was probed with a fragment of the actin gene, to detect intron and mature mRNA, and with U5 and U4 snRNA probes to monitor the immunoprecipitation with anti^{3}mG antibody. Deproteinized RNA from the mutant strain (lane 7) and wild type strain (lane 8) were also run on the gel to serve as markers for the RNA species being probed for.


Pre-Immune
Anti-3mG
Anti-Sm
RNA MARKER
RNA MARKER
WT
prp26
WT
prp26
WT
prp26
WT
1
2
3
4
5
6
7
8
mRNA
Intron
Linear Intron
snRNA7 (U5)
snRNA14 (U4)

Fi. 4. *In vitro* splicing reactions with *prp26* or wild type extract.

Splicing reactions with *prp26* extracts or wild type SS330 extracts were performed as described in Materials and Methods. Extracts were prepared from two mutant strains, an original isolate of *prp26 - ts* 45 and also from a strain obtained after two outcrosses *prp26*-5A-3C. The temperature and the time of incubation for the splicing reactions are indicated in the figure. The position of lariat intermediate (IVS*E2), lariat intron (IVS*), precursor (pre-mRNA), spliced product (mRNA) and exon1 (E1) are also indicated.


60′ 15°C
30′
60′ 15°C
10′
20′ 23°C
60′ 15°C
20′ 23°C
— IVS*E2
— IVS*
— pre-mRNA
— mRNA
— E1
prp26 –
prp26-5A-3c
SS330