IV SUMMARY AND CONCLUSIONS

Nuclear pre-mRNA splicing occurs via two trans-esterification reactions in the presence of ATP in a complex particle termed as the **spliceosome** The spliceosome is assembled from five U snRNAs and several trans-acting factors defined as *PRP* gene products The entire pathway requires a network of interactions To date, at least fifty different factors involved in splicing have been defined through various genetic screens and also by biochemical analysis However, the extensive network of protein-protein interactions and factors effecting conformational changes during assembly remain to be elucidated We have undertaken a molecular genetic analysis of the functional interactions between different *PRP* gene products involved in this pathway by the isolation and characterization of extragenic suppressors to ts *prp24-1* *prp24-1* was identified by Vijayraghavan et al (1989) from a screen of EMS induced temperature-sensitive mutants and this locus defines a requirement of the *PRP24* gene product, before the first step of splicing Three other alleles of *prp24* were identified as suppressors of U4/U6 snRNA destabilising mutations by Shannon and Guthrie (1991) Prp24 is an U6 snRNP binding protein and is involved in the assembly of U4/U6 snRNP We have screened for second-site suppressors of *prp24-1* to define factors interacting with the *PRP24* gene product The salient features of our analyses have been summarized here

Spontaneous revertants of *prp24-1* were obtained at 37^0C at a frequency of 10^{-8} Out of twenty-four revertants analyzed, four extragenic suppressors segregated conditional second-site suppressor loci - three of which were temperature-sensitive by themselves (r3, r6, and r9) and one of which conferred a cold sensitive phenotype (r2) The cold-sensitive second-site suppressor locus was distinct from the previously identified *prp3*, *prp4*, *prp5*, *prp11*, *prp16*, *prp17*, *prp18*, *prp19*, *prp21*, *prp22*, *prp24*, *prp27*, and *prp28* loci

Characterization of the temperature-sensitive second site suppressor of *prp24-1*

The three temperature-sensitive second site suppressors of *prp24-1* were allelic to each other Genetic complementation and transformation studies defined this locus as an allele of *prp21 1* and shall be referred to as *prp21-2* *prp21 1* was identified by Vijayraghavan et al (1989) from a screen of EMS induced temperature-sensitive mutants, and this mutant accumulates pre-mRNA at the non permissive temperature The *prp21* locus, *spp91-1* was also defined by Chapon and Legrain (1992) as an extragenic suppressor of *prp9* alleles We report here studies that define *prp21-2* as a second site suppressor of *prp24-1*

ts *prp21-2*, like *prp21-1* defines a requirement of the *PRP21* gene product before the first step of splicing as *prp21-2* cells accumulate pre-mRNA at the non-permissive temperature At the non-permissive temperature, *prp24-1* cells exhibit pleiotropic effects of pre-mRNA accumulation and reduction in the levels of U6 snRNA (Vijayraghavan et al 1989, Blanton et al 1992) Both these phenotypes are completely suppressed in the *prp21-2 prp24-1* cells The U snRNA levels are not perturbed in the *prp21-2* cells at the non-permissive temperature Thus a complete suppression of the phenotypes of *prp21-2* and *prp24-1* are observed in *prp21-2 prp24-1*

Genetic basis of suppression

prp21-2 is recessive to its wild type *PRP21* allele, however suppression is dominant as the diploids *prp21-2 prp24-1/PRP21 prp24-1* and *prp21-2 prp24-1/prp21-1 PRP24* are temperature resistant The suppression seen in *prp21-2 prp24-1*, could either be due to a functional overlap between *PRP21* and *PRP24* gene products, or suppression by bypassing the requirement for *PRP24*, or due to functional or physical interaction between Prp21 and Prp24 The suppression is unlikely to be due to a functional overlap between *PRP21* and *PRP24* as determined by overexpression studies This suppression does not occur bypassing the requirement of *prp24 -1* as the double mutant *prp21-2 ΔPRP24* is inviable

We have tested for the allele specificity of this interaction by generating double mutants with the two mutant alleles of *prp21* and four mutant alleles of *prp24* Suppression in *prp21 2 prp24 1* is **gene specific** and is **mutually allele specific** *prp21-2*, like *prp21-1* shows synthetic lethal interactions with *prp5*, *prp9*, and *prp11*, that are components of the prespliceosome However, no suppression or enhancement was observed when combined with *prp3*, or *prp4*, which are components of the U4/U6 snRNP or *prp8* a component of the U5 snRNP **Thus our genetic characterization defines an interaction between *prp21 -2* and *prp24-1* which could be either a physical or a functional interaction between the proteins** Prp21 is an U2 snRNP associated protein and Prp24 is an U6 snRNP binding protein The base pairing interaction between the U2 snRNA and the U6 snRNA required for the formation of the catalytic activation of the spliceosome is very well documented (Madhani and Guthrie 1994) This is the first report of an interaction between an U2 snRNA associated protein and an U6 snRNA associated protein

Mechanism of suppression.

An interaction between *PRP21* and *PRP24* (or the mutant *prp21-2* and *prp24-1*) gene products could occur as a direct physical interaction between the two proteins, or could occur in the pool of snRNPs, or during spliceosome assembly We have investigated all these three possibilities

We have assayed for physical interactions between Prp21 and Prp24 by employing the yeast two hybrid transactivation assay For these studies the wild type *PRP21* and *PRP24* ORFs were cloned independently as translational fusions to either the DNA binding domain of the Gal4 protein or to the transcriptional activation domain of Gal4 protein, present in the two hybrid vectors Since the suppression in *prp21-2 prp24-1* is allele specific we have tested whether the mutant prp21-2 and prp24-1 proteins interact directly with each other by this assay The mutant *prp21-2* and *prp24-1* loci were mapped, cloned and characterized They were subsequently cloned in the two hybrid transactivation assay vectors The mutation in *prp21-2* was a change in cysteine at position 164 to tyrosine

This mutation maps outside the conserved SURP modules seen in the SWAP family of proteins to which Prp21 belongs The *prp21-2* mutation like the *spp91-1* mutation however lies in the region of Prp21 which is conserved between the yeast and nematode Prp21 homologs Prp24 is an RNA binding protein with the characteristic RNA binding domains (RRMs) In *prp24-1* the mutation was mapped to two regions one which converts lysine 46 to arginine, in the conserved RNP2 of the RRM1, and the other which changes lysine 186 to glutamic acid and which lies in the RRM2 The position of this mutation is distinct from the other alleles of *prp24*, defined by Shannon and Guthrie (1991) In various pairwise combinations of the wild type and mutant proteins tested for transactivation of reporter strains, we determined that **the Prp21 and Prp24 wild type or mutant proteins do not interact with each other directly as assayed by the two hybrid transactivation assay** However the possibility of a transient interaction between the proteins in the presence of other splicing factors cannot be discounted

We have determined whether the suppression seen *in vivo* could be assayed *in vitro* to facilitate biochemical analysis Whole cell splicing extracts prepared from *prp21-2* and *prp24-1* were able to splice radiolabelled actin pre-mRNA *in vitro* Complete irreversible inactivation of the mutant proteins could be achieved *in vitro* by heat treatment of the extracts from the ts mutants at 39^0C for 7 minutes The allele-specific suppression seen *invivo* in *prp21-2 prp24-1* cells was also reflected *in vitro* as these extracts could splice *in vitro* under conditions which inactivated mutant prp21 or prp24 proteins The extracts from *prp21-1 prp24-1* could not splice actin pre-mRNA *in vitro* on similar heat treatment

We have assayed whether the U2 snRNP associated Prp21 protein and U6 snRNP associated Prp24 protein interact stably in the pool of snRNPs or during spliceosome assembly These studies employed immunoprecipitation analysis of U snRNAs by anti-Prp21 antisera and anti-Prp24 antisera We find that heat inactivated mutant prp21-2 protein does not stably associate with U2 snRNA, this defect is suppressed in the presence of the mutant prp24-1 protein Conversely, prp21-2 promoted association of prp24-1 to U6 snRNA However, we do not detect co-precipitation of U2 and U6 snRNAs in extracts

from the doubly mutant *prp21-2 prp24 1* strain Thus we **do not detect a stable interaction between the two proteins in terms of coprecipitation of associated U2 or U6 snRNAs** However, an interaction between the proteins that could occur in the spliceosome may not be manifested in the immunoprecipitation analysis

We have therefore assessed whether Prp21 and Prp24 coexist in the complexes that are assembly intermediates, which could represent points where suppression is likely to occur The kinetically ordered complexes can be resolved on non-denaturing polyacrylamide gels and the progression is from the prespliceosome, B to A2-1, an intermediate complex containing all the five U snRNPs, to the active spliceosome complex A (Cheng and Abelson 1987) Immunodepletion of selectively accumulated B and A2-1 spliceosome complexes by anti-Prp21 antisera showed that Prp21 in addition to being a component of prespliceosome, is also present in the spliceosome assembly intermediate A2-1 The A2-1 complex is formed when the tri snRNP-U4/U5/U6 assembles on the prespliceosome B While anti-Prp24 antisera immunoprecipitate some pre-mRNA, these antisera do not immunodeplete spliceosomal complexes Therefore, while we detect Prp21 in spliceosomal complex A immunodepletion studies do not clearly detect Prp24 in the same complex However, reports from other groups (Brow and Vidaver 1995, Ghetti et al 1995) our genetic analysis, and the lack of stable interaction between the two proteins indirectly suggest the presence of Prp24 in the A spliceosomal complexes

A kinetic analysis of spliceosome assembly using heat treated extracts from the revertant strain suggest that the transition of the prespliceosome B to A2-1 is a slow step These data, together with our immunodepletion studies suggest that suppression of *prp24 1* by *prp21-2* possibly occurs at this stage in the assembly pathway

We have addressed the functional requirement of Prp21 after formation of pre-spliceosome prp21-2 can be inactivated even in selectively accumulated A2-1 complexes Though Prp21 is present in A2-1 complex, its activity is not required for the progression of spliceosome assembly and splicing reactions However these experiments also suggested

that Prp21 function may be required for the conversion of prespliceosome B to A2-1 complex

Thus our characterization of the temperature sensitive second-site suppressor of *prp24-1 prp21-2*, defines a functional interaction between an U2 snRNP associated protein Prp21, and U6 snRNP associated protein Prp24 Our results suggest that this interaction between the two proteins occurs in the spliceosome