The EMBO Journal vol.13 no.4 pp.879-887, 1994

A dominant negative mutation in the conserved RNA helicase motif 'SAT' causes splicing factor PRP2 to stall in spliceosomes

Mary Plumpton[1], Margaret McGarvey and Jean D.Beggs[2]

Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, UK

[1]Present address: The Wellcome Research Laboratories, Department of Cell Biology, Langley Court, South Eden Park Road, Beckenham, Kent BR3 3BS, UK

[2]Corresponding author

Communicated by J.D.Beggs

To characterize sequences in the RNA helicase-like PRP2 protein of *Saccharomyces cerevisiae* that are essential for its function in pre-mRNA splicing, a pool of random *PRP2* mutants was generated. A dominant negative allele was isolated which, when overexpressed in a wild-type yeast strain, inhibited cell growth by causing a defect in pre-mRNA splicing. This defect was partially alleviated by simultaneous co-overexpression of wild-type *PRP2*. The dominant negative PRP2 protein inhibited splicing *in vitro* and caused the accumulation of stalled splicing complexes. Immunoprecipitation with anti-PRP2 antibodies confirmed that dominant negative PRP2 protein competed with its wild-type counterpart for interaction with spliceosomes, with which the mutant protein remained associated. The *PRP2-dn1* mutation led to a single amino acid change within the conserved SAT motif that in the prototype helicase eIF-4A is required for RNA unwinding. Purified dominant negative PRP2 protein had ~40% of the wild-type level of RNA-stimulated ATPase activity. As ATPase activity was reduced only slightly, but splicing activity was abolished, we propose that the dominant negative phenotype is due primarily to a defect in the putative RNA helicase activity of PRP2 protein.

Key words: dominant negative mutation/pre-mRNA splicing/PRP2/RNA helicase/yeast

Introduction

Nuclear pre-mRNA splicing, the process by which introns are accurately removed from primary transcripts, occurs within a large multicomponent RNA-protein complex, the spliceosome. Spliceosomes form by the ordered assembly onto the pre-mRNA of the U1, U2, U4/U6 and U5 small nuclear ribonucleoprotein particles (snRNPs) plus various non-snRNP proteins (reviewed in Green, 1991; Guthrie, 1991; Moore *et al.*, 1993). Following spliceosome assembly, splicing proceeds via two sequential transesterification reactions. The first reaction, or step 1, involves formation of a phosphodiester bond between the conserved guanosine at the 5′ end of the intron and the 2′ hydroxyl of the branchpoint adenosine (in *Saccharomyces cerevisiae* this is the most 3′ adenosine of the conserved UACUAAC sequence), which results in cleavage of the 5′ splice site. The products of step 1 are exon one, and intron-exon two in the form of a branched, lariat structure. In the second step the exons are joined, and cleavage at the 3′ splice site releases the lariat intron.

Although pre-mRNA splicing is an ATP-requiring process, the phosphates in the new phosphodiester bonds derive from the splice site phosphates of the pre-mRNA and not from exogenous ATP (Padgett *et al.*, 1984; Konarska *et al.*, 1985; Lin *et al.*, 1985). Indeed, the observation that nuclear pre-mRNA introns are removed via a similar chemical mechanism to that of autocatalytic group II introns led to the proposal that the two processes are evolutionarily and mechanistically related (Cech, 1985; Sharp, 1985). This forms the basis of the widely held belief that pre-mRNA splicing is RNA catalysed and questions the nature of the role of ATP in splicing.

Evidence for dynamic RNA-RNA interactions in spliceosomes (reviewed in Moore *et al.*, 1993) strongly suggests a central role for helicases in splicing. They are predicted to drive and monitor RNA conformational changes and displacement events at particular stages of the splicing reaction (Wassarman and Steitz, 1991). Such processes are likely to account for at least some of the ATP requirement of pre-mRNA splicing. Five yeast protein splicing factors, PRP2 (Chen and Lin, 1990), PRP5 (Dalbadie-McFarland and Abelson 1990), PRP16 (Burgess *et al.*, 1990), PRP22 (Company *et al.*, 1991) and PRP28 (Strauss and Guthrie, 1991), share a number of highly conserved amino acid motifs characteristic of ATP-dependent RNA helicases (DEAD- or DEAH-box proteins; reviewed in Koonin, 1991; Fuller-Pace and Lane, 1992; Schmid and Linder, 1992). Approximately 30 members of the DEAD/H-box family have been identified, but only eIF-4A (Ray *et al.*, 1985; Rozen *et al.*, 1990), human p68 protein (Hirling *et al.*, 1989), cylindrical inclusion protein of plum pox potyvirus (Lain *et al.*, 1990) and the human homologue of maleless (Lee and Hurwitz, 1993) have been demonstrated to have RNA helicase activity *in vitro*.

Detailed functional analysis of the helicase sequence motifs has been carried out only with the prototype DEAD-box protein, eIF-4A. From biochemical analyses of purified mutant forms of this translation factor, Pause and Sonenberg (1992) deduced functions for four of the most highly conserved motifs. Mutations within the N-terminal motifs I and II of eIF-4A, which are special forms of the previously defined ATPase motifs A and B (Walker *et al.*, 1982; Linder *et al.*, 1989), and a mutation in the C-terminal motif VI affected ATP binding and/or hydrolysis. On the other hand, mutation of motif III (SAT) caused complete abrogation of RNA helicase activity while ATPase activity increased (Pause and Sonenberg, 1992), indicating that integrity of the SAT motif is essential for coupling of ATP hydrolysis to RNA strand displacement activity.

Although purified PRP2 (Kim *et al.*, 1992) and PRP16 (Schwer and Guthrie, 1991) have RNA-stimulated ATPase activity *in vitro*, so far no RNA helicase activity has been demonstrated for any of the PRP proteins. Failure to demonstrate any RNA strand displacement activity for these putative helicases may be due to a stringent substrate specificity or the requirement for other factors to promote the activity of the purified proteins *in vitro*. The amino acid sequences of PRP2, PRP16 and PRP22 are very similar over ~450 residues and they function at sequential steps in the splicing process: PRP2 and PRP16 are required for steps 1 and 2 respectively (Lin *et al.*, 1987; Schwer and Guthrie, 1991) of the splicing reaction, and PRP22 promotes release of the spliced RNA products from the spliceosomes (Company *et al.*, 1991). PRP2 protein interacts only transiently with spliceosomes immediately prior to and during step 1 of the splicing reaction (King and Beggs, 1990). Similarly, PRP16 protein interacts transiently with spliceosomes at step 2 (Schwer and Guthrie, 1991). It has been speculated that PRP16 protein may regulate the fidelity of the second step of splicing, possibly via an ATP-dependent kinetic proofreading mechanism that discards aberrant lariat intermediates (Burgess *et al.*, 1990; Schwer and Guthrie, 1991; Burgess and Guthrie, 1993).

To characterize sequences in the PRP2 protein that are essential for its function in pre-mRNA splicing and, in particular, that influence its interactions with spliceosomal factors, we sought dominant negative *PRP2* mutants. The principle behind this approach is that mutations in *PRP2* that interfere with (e.g. by hyperstabilizing) spliceosomal interactions may prevent the release of PRP2 protein from splicing complexes and therefore confer a dominant negative phenotype (i.e. cause a dominant inhibitory effect over the wild-type protein; Herskowitz, 1987). In the case of a protein with a putative RNA destabilizing activity this may be a particularly informative type of mutation.

We report the isolation of a dominant negative *PRP2* allele which, when overexpressed, causes a defect in pre-mRNA splicing. Consistent with our prediction, the mutant PRP2 protein remains associated with stalled splicing complexes. The mutation responsible for the dominant negative phenotype causes a single amino acid change within the highly conserved helicase motif 'SAT'. This is the first time that the functional importance of this motif has been demonstrated in any of the DEAD- or DEAH-box splicing factors. We show that the purified dominant negative mutant protein retains the ability to hydrolyse ATP, albeit at a reduced rate. This suggests that the primary defect is probably not in the ATPase activity, and by analogy with eIF-4A, a defect in an RNA strand displacement activity may be responsible for the dominant negative phenotype.

Results

Isolation and in vivo characterization of dominant negative PRP2 mutants

An important requirement for the isolation and propagation of dominant negative mutants is a regulated expression system. Plasmid pBM-PRP2 (King and Beggs, 1990) contains the PRP2 coding region fused to the *GAL1* promoter, which is inducible by galactose and repressed by growth in the presence of glucose. pBM-PRP2 DNA was subjected to random chemical mutagenesis by treatment with

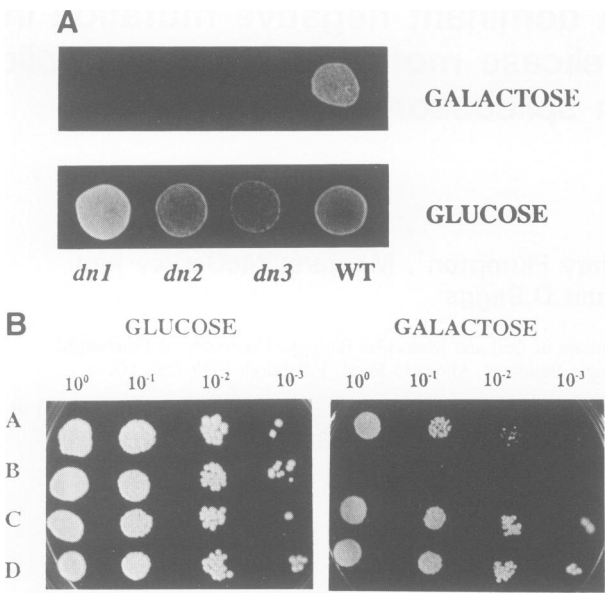

Fig. 1. Isolation of dominant negative PRP2 mutants. (**A**) Three independent isolates fail to grow on galactose medium. Following colony purification, three mutant isolates (*dn1*, *dn2* and *dn3*), identified in the screen for dominant negative mutants, and as a control, S150-2B cells harbouring pBM-PRP2 (WT) were grown overnight in YMMCas (repressing conditions) at 30°C. Cells were harvested, resuspended in dH_2O to ~10^6 cells/ml, spotted onto either selective YMGRCas (galactose) or selective YMMCas (glucose) agar and incubated for 60 h at 30°C. (**B**) Simultaneous overproduction of wild-type PRP2 protein and $PRP2^{dn1}$ protein in S150-2B cells partially restores growth. S150-2B cells co-transformed with either pBM-$PRP2^{dn1}$ and pFL45-PRP2 (line A) or pBM-$PRP2^{dn1}$ and pFL45 (line B), pBM-PRP2 and pFL45-PRP2 (line C) or pBM-PRP2 and pFL45 (line D) were grown overnight in YMMCas at 30°C, spun down and resuspended in dH_2O to 2×10^5 cells/ml. Drops of these and of 10-fold serial dilutions were spotted onto selective YMMCas (glucose) or YMGRCas (galactose) plates and incubated for 60 h at 30°C.

hydroxylamine, then introduced directly into the *S.cerevisiae* strain S150-2B. Transformants obtained on glucose-containing medium (repressing condition) were screened for those which failed to grow on galactose (inducing) medium. Out of 800 tested, three independent isolates were obtained that were able to grow on glucose but not on galactose at all temperatures (18°C, 23°C, 30°C, 34°C and 36°C) whereas cells carrying untreated pBM-PRP2 were able to grow on both glucose and galactose media (Figure 1A; growth at 30°C).

When the three mutant isolates were cured of the plasmid DNA by growth in non-selective glucose medium, in all cases the cured cells lost the dominant negative phenotype, indicating that it was plasmid-mediated. This was confirmed by recovering plasmid DNA from the three S150-2B dominant negative isolates (the plasmids were named pBM-$PRP2^{dn1}$, pBM-$PRP2^{dn2}$ and pBM-$PRP2^{dn3}$) and transforming other S150-2B cells. All three plasmids conferred a galactose-sensitive growth phenotype on the transformed cells.

To probe the mechanism for the dominance exhibited by the *PRP2-dn1* allele, the ability of wild-type *PRP2* to overcome the dominant negative phenotype when expressed at high level was tested. The strain S150-2B was co-transformed with pBM-$PRP2^{dn1}$ and pFL45-PRP2, the latter providing high level expression of wild-type *PRP2* from its own promoter by virtue of the high copy number

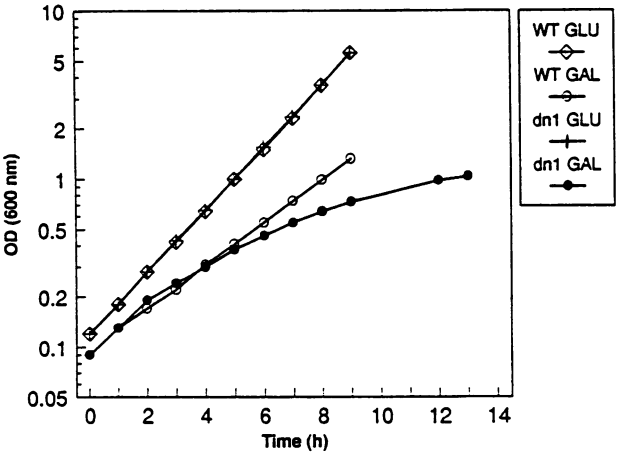

Fig. 2. Growth curves of cells containing pBM-PRP2 or pBM-PRP2^{dn1}. Mid-log phase cultures of S150-2B cells carrying pBM-PRP2 (WT) or pBM-PRP2^{dn} (dn1) were spun down (time 0) and half of the cells were returned to YMMCas (GLU; repressing conditions), while the other half was shifted into YMGRCas (GAL; inducing conditions). The cultures were diluted to maintain logarithmic growth which was monitored by measuring OD_{600nm}. N.B. The data points for WT GLU and dn1 GLU are superimposed.

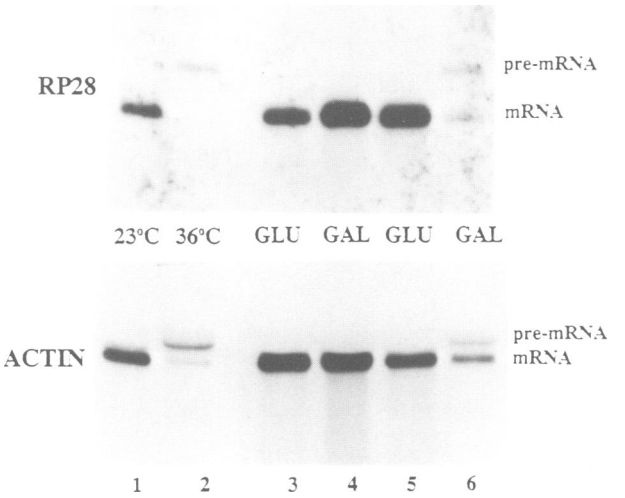

Fig. 3. Overproduction of PRP2^{dn1} protein in S150-2B cells inhibits splicing. Total RNA was extracted from S150-2B cells carrying pBM-PRP2 or pBM-PRP2^{dn1} grown under repressing conditions (lanes 3 and 5) or inducing conditions (lanes 4 and 6), and from a *prp2-1* temperature-sensitive strain (DJY36) grown at the permissive temperature, 23°C (lane 1), or shifted to the non-permissive temperature, 36°C (lane 2) for 2 h. RNA (30 μg) was denatured and fractionated on a 1.4% (w/v) agarose gel, blotted onto Hybond-N and hybridized to ^{32}P-labelled DNA fragments encoding actin or rp28. The positions of unspliced precursor RNA (pre-mRNA) and spliced messenger RNA (mRNA) are marked.

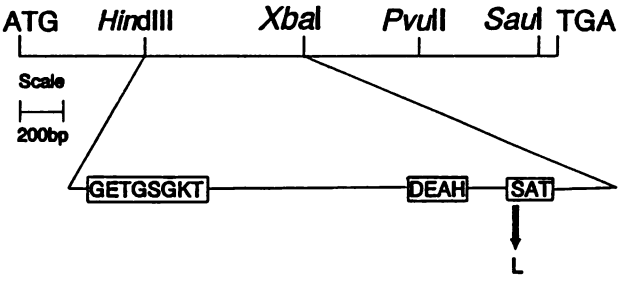

Fig. 4. Map of the *PRP2* gene with the central 777 bp region showing conserved amino acid sequence motifs enlarged. The *PRP2-dn1* mutation present in pBM-PRP2^{dn1} was mapped as a point mutation causing a serine to leucine change of amino acid 378 in the conserved SAT motif.

2μ origin of replication. Figure 1B shows the growth of various co-transformants on glucose or galactose medium. Unlike cells harbouring pBM-PRP2^{dn1} plus pFL45 vector alone (row B), which exhibited the dominant phenotype, cells harbouring pBM-PRP2^{dn1} plus pFL45-PRP2 were able to grow on galactose medium (row A), although not quite as well as control cells carrying only wild-type pBM-PRP2 (rows C and D). Thus overproduction of wild-type PRP2 protein partially suppressed the dominant inhibitory phenotype, indicative of competition between mutant and wild-type forms of PRP2 for a cellular factor(s).

Figure 2 shows growth curves for S150-2B cells harbouring pBM-PRP2 or pBM-PRP2^{dn1}. pMB-PRP2^{dn1} had no effect on growth in glucose medium, however, 5–6 h following transfer to galactose medium the growth rate of cells carrying the mutant plasmid declined dramatically. Splicing extract was prepared from S150-2B cells harbouring pBM-PRP2 or pBM-PRP2^{dn1} 6 h after transferring to inducing conditions, and proteins were Western blotted and probed with anti-PRP2 antibodies. The wild-type and mutant forms of PRP2 protein were strongly overproduced (~50-fold), compared with the levels in glucose-grown cells (data not shown), demonstrating that the growth defect in galactose medium correlated with the overproduction of PRP2^{dn1} protein.

If, as the co-transformation experiment described above suggests, PRP2^{dn1} protein competes with and functionally blocks its wild-type counterpart, overproduction of the mutant form would be expected to cause a defect in pre-mRNA splicing. RNA was prepared from S150-2B cells, carrying pBM-PRP2 or pBM-PRP2^{dn1}, which had been grown in glucose medium, or transferred to galactose medium for 6 h. Figure 3 shows Northern blots of transcripts from the *RP28* and *ACT1* genes, each of which contains a single intron. For comparison, RNA prepared from a recessive heat-sensitive *prp2-1* mutant (strain DJY36) grown at the permissive temperature (23°C) or incubated at the non-permissive temperature (36°C) for 2 h was also analysed (lanes 1 and 2). For the *prp2-1* mutant, a severe splicing defect was apparent at 36°C (lane 2). Unspliced pre-mRNA also accumulated in the dominant negative mutant grown under inducing conditions (lane 6) but not when grown under repressing conditions (lane 5), whereas overexpression of wild-type *PRP2* had no effect on pre-mRNA splicing (lane 4). This indicated that the growth defect caused by overproduction of PRP2^{dn1} protein was the result of a defect in pre-mRNA splicing.

These experiments were also carried out to analyse the effects of the other two mutant plasmids, pBM-PRP2^{dn2} and pBM-PRP2^{dn3}, with identical results (data not shown).

The PRP2-dn mutations all cause a single serine to leucine substitution within the highly conserved SAT motif

To localize the *PRP2-dn1* mutation and avoid the necessity of sequencing the entire *PRP2* gene, chimeras were made between the dominant *PRP2-dn1* allele and wild-type *PRP2*. Three non-overlapping regions (*Hin*dIII–*Xba*I, *Xba*I–*Pvu*II and *Pvu*II–*Sau*I restriction fragments; Figure 4) of the wild-type sequence were replaced by the corresponding sequence

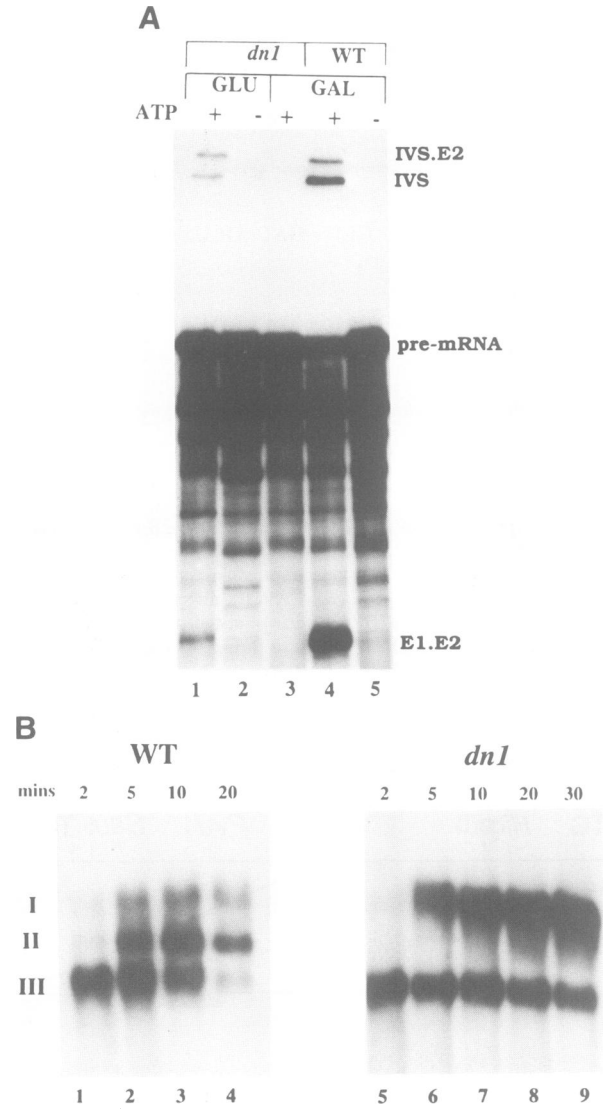

Fig. 5. Overproduction of PRP2^{dn1} protein inhibits pre-mRNA splicing and spliceosome formation *in vitro*. (**A**) Splicing extracts were prepared from S150-2B cells carrying pBM-PRP2^{dn1} and grown under repressing conditions (lanes 1 and 2) or inducing conditions for 6 h (lane 3) or from S150-2B carrying pBM-PRP2 grown under inducing conditions for 6 h (lanes 4 and 5). Splicing reactions (10 μl) containing radiolabelled rp28 pre-mRNA and 50% (v/v) splicing extract were incubated for 30 min at 25°C either in the presence (lanes 1, 3 and 4) or absence (lanes 2 and 5) of ATP. RNA was recovered and fractionated on a 6% (w/v) denaturing polyacrylamide gel. IVS.E2, lariat intron–exon 2 intermediate species; IVS, lariat excised intron; pre-mRNA, rp28 substrate RNA; E1.E2, spliced exons. (**B**) For analysis of splicing complex formation, 5 μl aliquots of the dn1-GAL or WT-GAL splicing reactions shown in panel A (lanes 3 and 4) were incubated for the times indicated (2, 5, 10, 20 and 30 min), followed by fractionation on a composite 0.25% (w/v) agarose, 3% (w/v) polyacrylamide non-denaturing gel. Complexes were designated I, II and III according to Pikielny *et al.* (1986).

from the mutant allele and the chimeric genes were expressed in S150-2B cells. The region of *PRP2-dn1* responsible for the dominant phenotype was mapped to within the *Hin*dIII–*Xba*I region. This region encodes the conserved motif sequences common to the DEAH proteins.

When this entire 777 bp region was sequenced, only a single mutation (C to T at nucleotide 1336) was identified which causes a serine to leucine substitution at amino acid 378 within the highly conserved SAT motif (Figure 4). We confirmed that this was the cause of the dominant negative phenotype by introducing the single point mutation into the wild-type *PRP2* gene by site-directed mutagenesis. When the *Hin*dIII–*Xba*I regions of the *PRP2-dn2* and *PRP2-dn3* alleles were sequenced, each contained only a single point mutation, exactly the same as the *PRP2-dn1* mutation, causing the S378L substitution. Therefore out of 800 transformants screened, three independent isolates contained the same dominant inhibitory mutation.

PRP2^{dn1} protein inhibits splicing in vitro causing the accumulation of inactive splicing complexes

To investigate the effect of PRP2^{dn1} protein on RNA splicing *in vitro*, splicing assays were carried out using extracts prepared from S150-2B cells in which expression of *PRP2-dn1* was induced or repressed, and as a control, from cells overexpressing wild-type *PRP2* from the same inducible promoter. Consistent with the *in vivo* data, splicing extract prepared from *PRP2-dn1* cells grown under non-inducing conditions (dn1–GLU) was active (Figure 5A, lane 1), but extract prepared from the same strain grown under inducing conditions (dn1–GAL) was inactive (lane 3), whereas extract prepared from cells overproducing the wild-type protein (WT–GAL) was active (lane 4). Thus a high level of PRP2^{dn1} protein abolished splicing *in vitro* while a similar amount of the wild-type protein was not detrimental.

To assess the effects of PRP2^{dn1} on spliceosome assembly, extracts containing a high level of either wild-type PRP2 or PRP2^{dn1} protein were incubated under *in vitro* splicing conditions and the complexes that formed on the pre-mRNA were analysed at intervals by native gel electrophoresis (Figure 5B). The gel system used was essentially that described by Pikielny *et al.* (1986) which resolves three discrete complexes designated I, II and III in order of increasing electrophoretic mobility. In this system, pre-mRNA assembles first into complex III, then complex I and the final complex, II, contains the intermediates and products of the splicing reaction. A typical time course of complex assembly with wild-type extract is shown in lanes 1–4. In contrast, in the PRP2^{dn1} extract (lanes 5–9), complexes III and I accumulated to high levels and conversion to complex II was completely blocked. There were no signs of complex disassembly, even after 30 min (lane 9), thus the release of spliceosomal components assembled into this 'stalled' complex apparently did not occur.

Experiments in which extracts overproducing PRP2^{dn1} or wild-type PRP2 protein were mixed in different ratios, and tested for complementation of the splicing defect of a heat-inactivated prp2-1 extract, showed that the activity of wild-type PRP2 protein was partially inhibited by an equal amount and completely inhibited by a 3- to 8-fold excess of PRP2^{dn1} protein (data not shown), suggesting that PRP2^{dn1} protein competes with wild-type PRP2 protein when present in similar amounts, and that it might act in a co-dominant (or at least partially dominant) manner. This would imply that the mechanism for the dominant phenotype involves sequestration of essential spliceosomal components. Such a mechanism is also compatible with the very high level accumulation of complex I in the presence of the mutant protein.

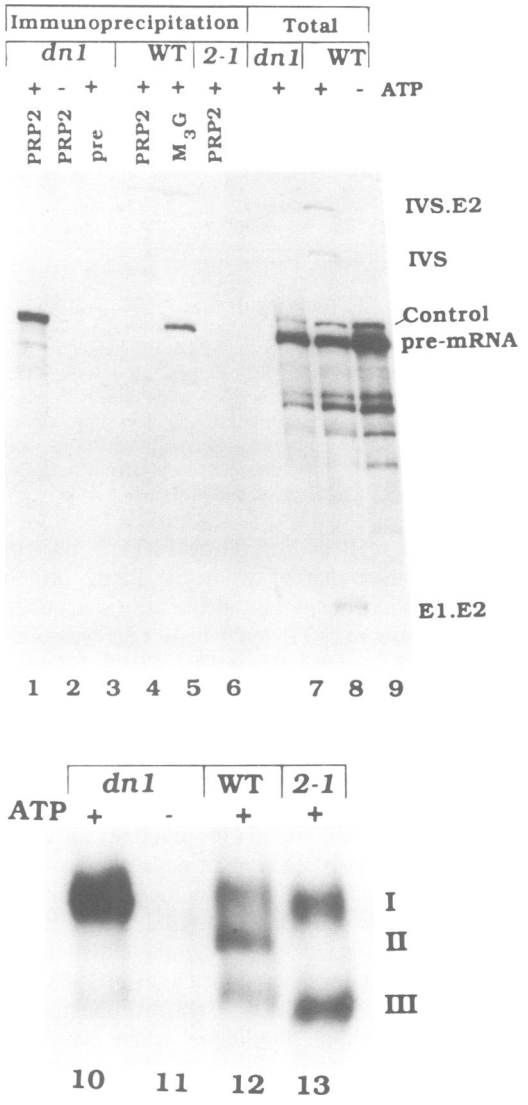

Fig. 6. Anti-PRP2 antibodies co-immunoprecipitate splicing complexes with PRP2^{dn1} protein. *In vitro* splicing reactions (20 μl) were carried out using rp28 pre-mRNA plus an intronless control RNA and 8 μl of dn1-GLU extract mixed with 2 μl of extract containing overproduced PRP2^{dn1} (dn1) or wild-type PRP2 (WT) protein. (To avoid saturation of anti-PRP2 antibodies by the superfluous wild-type PRP2 or PRP2^{dn1} protein present in galactose-grown cells, a titration experiment was carried out and 2 μl was determined to be the minimum volume of dn1-GAL extract required to inhibit the activity of dn1-GLU extract; data not shown.) For comparison, a heat-inactivated prp2-1 extract was analysed (2-1). RNA was extracted from 3 μl to measure splicing activity (lanes 7–9) and 5 μl were analysed for splicing complex formation (lanes 10–13) as in Figure 5. Immunoprecipitation was carried out with 10 μl of each reaction using anti-PRP2 antibodies (lanes 1, 2, 4 and 6), pre-immune serum (lane 3), or anti-trimethylguanosine antibodies (lane 5). IVS.E2, lariat intron–exon 2 intermediate species; IVS; lariat intron product; E1.E2, spliced exons. N.B. In lane 10 very little complex III is present, unlike the result with dn1-GAL extract alone (Figure 5B) (or with prp2-1 extract; lane 13). Possibly the presence of dn1-GLU extract with the dn1-GAL extract (as in lane 10) increases the convertion of complex III to complex I.

PRP2^{dn1} protein associates with splicing complexes

The interaction of PRP2^{dn1} protein with splicing complexes was examined directly by immunoprecipitation with anti-PRP2 antibodies (Figure 6). In this way we have demonstrated previously that wild-type PRP2 protein interacts only transiently with spliceosomes (King and Beggs, 1990). If PRP2^{dn1} protein is indeed retained in 'stalled' complexes, thus denying entry of wild-type PRP2 protein, ^{32}P-labelled pre-mRNA should be strongly co-immunoprecipitated by PRP2-specific antibodies from splicing reactions containing PRP2^{dn1} protein, in contrast to relatively inefficient co-precipitation of complexes associated with wild-type protein. Analysis of the RNA species (Figure 6, lanes 7, 8 and 9) and the splicing complexes formed during such reactions (lanes 10–13) confirmed that in the presence of PRP2^{dn1} protein, splicing was inhibited due to a block in the transition of complex I to complex II. This resembles the defect in spliceosome assembly observed during incubation with heat-inactivated *prp2-1* extract (lane 13).

Anti-PRP2 antibodies strongly immunoprecipitated pre-mRNA-associated complexes from the dominant negative reaction (lane 1) indicating that PRP2^{dn1} protein was indeed stably associated with spliceosomes. This association of PRP2^{dn1} with pre-mRNA was dependent on the formation of complexes and was not observed in the absence of ATP (lanes 2 and 11), nor with an intronless control transcript which would not be assembled into spliceosomes (present in all samples). In contrast, immunoprecipitation of labelled RNA species from the wild-type splicing reaction was almost undetectable (lane 4) (a weak signal was detected with longer exposure times), whereas strong precipitation of pre-mRNA was achieved using anti-trimethylguanosine antibodies which precipitate spliceosomes through association with the spliceosomal snRNAs. As we have shown previously, heat-inactivated prp2 protein produced from the recessive temperature-sensitive *prp2-1* allele fails to associate detectably with complexes (lane 6; King and Beggs, 1990). Therefore, although both the dominant and the recessive mutations cause complexes to be stalled prior to the formation of active spliceosomes, the PRP2^{dn1} protein interacts stably with splicing complexes, whereas heat-inactivated prp2 protein of the temperature-sensitive *prp2-1* strain apparently does not.

Purification of PRP2 and PRP2^{dn1} proteins

To facilitate purification of PRP2 protein, the *PRP2* gene was modified to extend the amino-terminus of the protein, including a run of six contiguous histidine residues. The histidine-tagged wild-type PRP2 protein was demonstrated to be functional *in vivo* by introduction of the plasmid-encoded gene into the *prp2-1* strain DJY36 where it suppressed the heat-sensitive growth defect caused by *prp2-1* mutation. Similarly, the histidine-tagged PRP2^{dn1} protein retained the ability to cause the dominant negative phenotype; S150-2B cells expressing histidine-tagged *PRP2-dn1* grew on glucose but not on galactose medium (data not shown).

The tagged proteins were purified by nickel affinity chromatography (Hoffmann and Roeder, 1991), followed by affinity chromatography on poly(U)–agarose (Kim *et al.*, 1992). This simple two-step procedure produced a 1500-fold purification of wild-type PRP2 protein and a 13% overall yield. No contaminating proteins could be detected by SDS–polyacrylamide gel electrophoresis and silver staining of the purified protein (Figure 7). The method was highly reproducible and the behaviour of mutant and wild-type proteins throughout the procedure was similar with respect to stability, yield and binding/elution properties.

ATPase assays

The ATPase activities of the purified wild-type PRP2 and PRP2^{dn1} proteins were compared (Figure 8). The purified

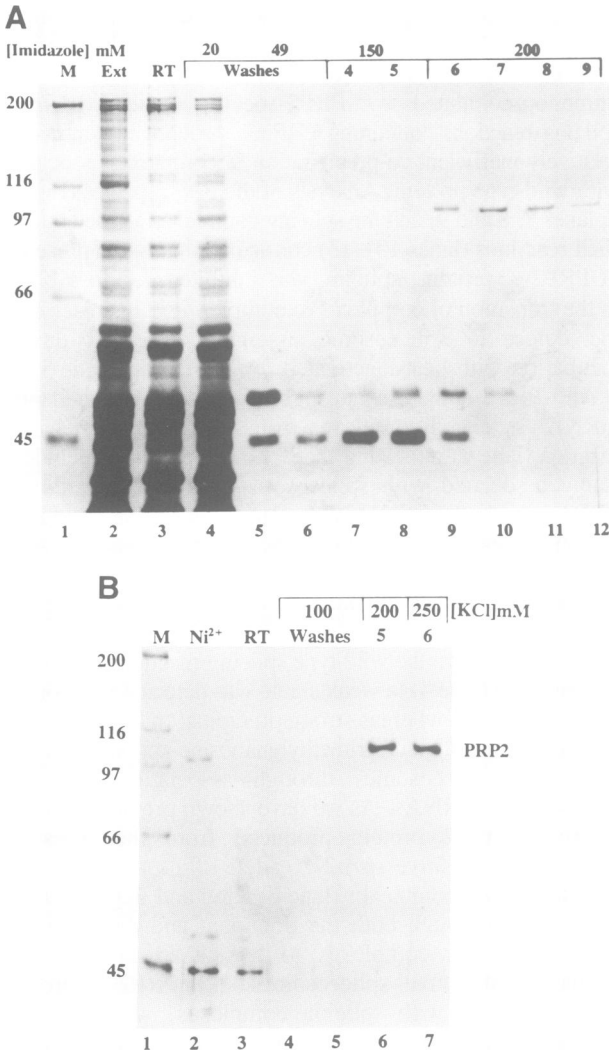

Fig. 7. Two-step purification of polyhistidine-tagged PRP2 protein. Aliquots of fractions from the two-step purification of polyhistidine-tagged PRP2 protein were analysed by electrophoresis through a 7.5% SDS−polyacrylamide gel and silver staining. (**A**) Step I: nickel chelating column. Lane 1, marker proteins with sizes indicated in kDa; lane 2, unfractionated extract (1 μl) prepared from S150-2B cells overproducing polyhistidine-tagged PRP2 protein; lane 3, run-through (1 μl) from the nickel column; lane 4, aliquot (30 μl) of first 1 ml fraction of 20 mM imidazole wash; lanes 5 and 6, aliquots (30 μl) of first and final 1 ml fractions respectively of 49 mM imidazole wash; lanes 7 and 8, aliquots (30 μl) of elution fractions 4 and 5 (150 mM imidazole); lanes 9−12, aliquots (30 μl) of elution fractions 6−9 (200 mM imidazole). (**B**) Step II: poly(U)−agarose column. Lane 1, marker proteins with sizes indicated in kDa; lane 2, aliquot (30 μl) of pooled fractions 5−12 from the nickel column; lane 3, aliquot (30 μl) of run-through following passage over poly(U)−agarose column; lanes 4 and 5, aliquots (30 μl) of first and final 1 ml fractions of 100 mM KCl wash; lanes 6 and 7, aliquots (30 μl) of 0.45 ml elution fractions 5 and 6 (200 and 250 mM KCl).

wild-type PRP2 protein showed some ATPase activity in the absence of RNA which was stimulated 10-fold by the addition of poly(U) RNA. (Unlike a mock purification fraction from cells lacking a histidine-tagged PRP2 protein, which possessed negligible activity even in the presence of RNA; data not shown.) The mutant protein was observed to have decreased ATPase activity compared with the wild-type protein and was stimulated only 5-fold by the addition of poly(U) RNA. In this experiment, the overall rate of

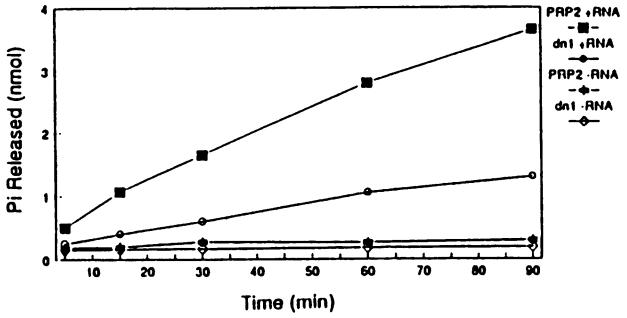

Fig. 8. PRP2^{dn1} protein has slightly reduced RNA-stimulated ATPase activity compared with wild-type PRP2 protein. To analyse ATPase activity of PRP2 protein, 60 ng of purified wild-type PRP2 protein (filled square, asterisk) or dominant negative protein (open circles, open diamond) were incubated in the presence (filled square, open circle) or absence (asterisk, open diamond) of poly(U) RNA in 20 μl reaction mixes at 25°C for the times indicated.

RNA-stimulated ATP hydrolysis by PRP2^{dn1} protein was 2.8-fold lower than that of wild-type PRP2 protein. In another two, independent but identical, experiments the observed decrease in ATP hydrolysis rate was 2.2- and 2.5-fold. Therefore, on average, the *PRP2-dn1* mutation conferred a 2.5-fold reduction in ATP hydrolysis.

Discussion

This paper describes the isolation and characterization of a dominant negative PRP2 mutant protein that was generated by random mutagenesis. Dominant negative mutants have proved useful in studies of other multicomponent systems such as the cell cycle (Mendenhall *et al.*, 1988; Fleig *et al.*, 1992) and signal transduction, for example the interactions of cellular Ras proteins (Feig and Cooper, 1988) and G-protein signalling (Leberer *et al.*, 1992). Although two other splicing mutants exhibit dominance when overexpressed (Miraglia *et al.*, 1991; Schwer and Guthrie, 1992), this is the first time that a search for dominant inhibitory splicing mutants has been carried out.

From a screen of 800 yeast transformants harbouring a mutagen-treated plasmid expressing a galactose-regulated *PRP2* gene (pBM-PRP2), three independent galactose-sensitive isolates were obtained. The fact that all three mutants carried the same mutation probably reflects a combination of the stringent phenotype demanded in the screen and the specificity of the chemical mutagen used. Hydroxylamine specifically induces transition mutations, thus the variety and number of amino acid substitutions that can be induced by hydroxylamine in a given sequence is limited.

In the screen for dominant mutants, high level expression from the *GAL1* promoter was employed to increase the likelihood that mutant proteins would compete effectively with wild-type PRP2 (Herskowitz, 1987). However, as the growth inhibition caused by pBM-PRP2^{dn1} was only partially alleviated by co-overproduction of wild-type PRP2 from its homologous promoter on a high copy number 2μ plasmid, the level of expression of *PRP2-dn1* from the *GAL1* promoter is probably higher than necessary to effect the dominant phenotype. It was demonstrated that the reduced growth rate of cells overproducing PRP2^{dn1} protein was the result of a pre-mRNA splicing defect. The dominant negative effect on splicing caused by PRP2^{dn1} protein was reproduced in an *in vitro* splicing system, with the formation

of stalled spliceosomes. Titration experiments indicated that the splicing activity of wild-type PRP2 protein was blocked by similar amounts of PRP2^{dn1} protein, indicating that the mutant protein is co-dominant or partially dominant *in vitro*.

One mechanism for dominance, of importance for studying interactions between proteins, involves competition between the mutant gene product and its wild-type counterpart for binding to an essential cellular factor (Herskowitz, 1987). The ability of high levels of the wild-type PRP2 protein to partially overcome the PRP2^{dn1} phenotype favours such a competitive mechanism for dominance. A model in which mutant and wild-type PRP2 molecules compete for binding to spliceosomes is supported by the observed co-immunoprecipitation of pre-mRNA-associated complexes from *in vitro* splicing reactions using PRP2-specific antibodies. PRP2^{dn1} protein was indeed shown to be strongly associated with inactive spliceosomes whereas wild-type and heat-inactivated prp2 proteins were not. Thus the molecular basis for the dominant phenotype was confirmed: PRP2^{dn1} associates with splicing complexes in competition with wild-type PRP2 protein. This interaction is either irreversible, or it is prolonged, delaying access of wild-type PRP2 protein. In contrast, the recessive nature of the *prp2-1* mutant observed *in vivo*, is explained by failure of the heat-inactivated prp2 molecules to associate with complexes, leaving the PRP2 binding site available for entry of the wild-type protein (King and Beggs, 1990).

The independent isolation of three mutant clones carrying the S378L (SAT to LAT) substitution attests to the functional importance of the SAT domain which, in conjunction with the six other helicase signatures, is found not only within the DEAD/H proteins but more widely in members of a vast superfamily of demonstrated and presumptive DNA and RNA helicases delineated by Gorbalenya *et al.* (1989). The closely related DEAD/H proteins form a subdivision of this superfamily (SFII) which includes proteins of bacteria, eukaryotes, RNA and DNA viruses. Within the DEAD and DEAH subfamilies the SAT residues in motif III are completely conserved except in the splicing factor PRP28 which encodes the variant TAT at this position (Schmid and Linder, 1992).

Intriguing parallels can be made between the PRP2^{dn1} results presented here and the analysis of mutants of the *RAD3* gene of *S.cerevisiae* which encodes a well-studied ATP-dependent DNA−DNA and DNA−RNA helicase (reviewed in Deschavanne and Harosh, 1993). RAD3 as a more distant member of the helicase superfamily II contains the variant SGT instead of SAT in motif III (Gorbalenya *et al.*, 1989). One mutant allele, *rad3-2* which causes a UV-sensitive phenotype, results from a single point mutation causing the amino acid substitution SGT to SRT (Naumovski and Friedberg, 1986). Significantly, overexpression of the mutant rad3-2 protein rendered wild-type yeast cells partially UV-sensitive. The susceptibility of the motif III sequence to dominant inhibitory mutations demonstrates the functional importance of this motif, implying that this function might be conserved throughout the helicase superfamily.

The function of the SAT motif has been probed only for the prototype of the DEAD family, eIF-4A, in which mutation of SAT to AAA caused complete abrogation of the RNA helicase activity of the purified protein *in vitro*, while the rate of ATP hydrolysis was increased 2.4-fold with respect to the wild-type level (Pause and Sonenberg, 1992). Therefore for eIF-4A, the SAT residues are critical for RNA strand displacement but not for ATP hydrolysis; these activities are apparently uncoupled in the mutant protein. Therefore the SAT residues may be involved in the transmission of energy derived from ATP hydrolysis to drive the RNA strand displacement.

In a recent study of PRP16 protein, which shares many biochemical characteristics with PRP2, a number of mutant *prp16* alleles were isolated that suppress the splicing defect of introns with mutant branchpoint sequences. Three of the purified mutant prp16 proteins when assayed for ATPase activity *in vitro* displayed decreases of 3- to 20-fold compared with the wild-type protein, yet showed no loss of splicing activity (Burgess and Guthrie, 1993). By comparison, the 2.5-fold decrease in ATPase activity caused by the *PRP2-dn1* mutation is modest, especially considering the severe splicing defect that results. This suggests that another activity may also be defective, perhaps one which is coupled to ATP hydrolysis.

As the PRP2^{dn1} and wild-type PRP2 proteins were purified with similar yield in the two-step procedure, the S378L mutation apparently has no effect on the stability of the protein nor on its ability to bind to poly(U) RNA during purification. It therefore seems unlikely that the 2.5-fold decrease in the RNA-stimulated ATPase activity of PRP2^{dn1} compared with the wild-type protein results from a reduction in RNA binding by PRP2^{dn1}. Indeed, the PRP2^{dn1} protein has been demonstrated to retain the ability to bind to pre-mRNA within the more physiological context of the spliceosome (Teigelkamp *et al.*, 1994). Although a decrease in ATP binding cannot be ruled out, the fact that mutation of the SAT residues of eIF-4A increased ATP binding (Pause and Sonenberg, 1992) suggests that the interaction of ATP at the ATP binding domain is not inhibited by mutations within this motif. Thus it is more likely that the change in the rate of ATP hydrolysis is due to an indirect effect, such as failure to couple RNA binding to ATP hydrolysis.

Attempts to date to demonstrate RNA unwinding activity for either PRP2 or PRP16 proteins have failed (Schwer and Guthrie, 1991; Kim *et al.*, 1992; M.McGarvey and J.D.Beggs, unpublished data). There are at least three possible explanations for this: (i) these proteins may not be classical RNA helicases, but perhaps utilize energy derived from ATP hydrolysis for an activity that is mechanistically distinct from that exhibited by DEAD protein helicases, (ii) they may be RNA helicases with stringent substrate specificity, acting only on RNA helices with a particular secondary structure or sequence and/or (iii) they may require *trans*-acting auxiliary factors not present in the purified preparations. Indeed, it is relevant that in UV-crosslinking experiments, PRP2 protein was observed to bind only to pre-mRNAs that were assembled into spliceosomes and were capable of being spliced; in addition, the interaction of PRP2 protein with at least one other spliceosome component was apparent (Teigelkamp *et al.*, 1994). Also, the auxiliary factor eIF-4B is required for helicase activity of eIF-4A (Rozen *et al.*, 1990) and the *Escherichia coli* protein, DbpA, displays ATPase activity only in the presence of bacterial 23S rRNA (Fuller-Pace *et al.*, 1993).

Our results are consistent with a scenario in which PRP2^{dn1} protein enters spliceosomes and interacts with one or more target RNA(s) but is unable to accomplish an RNA displacement event. This defect may inhibit a conformational

change required for step 1 of the splicing reaction, and consequently release of PRP2^{dn1} protein from the inactive complex is curbed.

As both the wild-type and PRP2^{dn1} proteins have been shown by UV-crosslinking to contact the pre-mRNA in spliceosomes (Teigelkamp *et al.*, 1994) this is a likely target for an RNA conformational change or displacement activity promoted by PRP2 protein, which may affect one of the many interactions between the pre-mRNA and snRNAs in spliceosomes. The dominant negative *PRP2* mutant now provides a powerful tool to characterize further the interactions of PRP2 protein with the pre-mRNA and other spliceosomal factors, by isolating either mutant or high copy number suppressors which may encode PRP2 ligands, and through biochemical analyses of the stalled splicing complex. Applied to other splicing factor genes, this dominant negative approach could facilitate the analysis of interactions at each step of the spliceosome cycle and should be a particularly valuable strategy for genetically intractable mammalian systems (Herskowitz, 1987).

Materials and methods

Strains, media and plasmids

S.cerevisiae strains used were S150-2B (*MAT***a**, *ura3-52, leu2-3,-112, trp1-289, his3*-Δ1; Baldari *et al.*, 1987), DJY36 (*MAT***a**, *prp2-1, ura3-52, ade*; D.J.Jamieson, Dundee) and DJY85 (*MAT***a**/α, *prp2-1/prp2-1, ura3/ura3, ade1/ADE1, ade2/ade2, trp1/TRP1, his3/HIS3, tyr1/TYR1, lys2-801/LYS2, can1/CAN*1; D.J.Jamieson, Dundee). Yeast transformations were carried out following the method of Ito *et al.* (1983). Selective, non-inducing yeast medium was YMMCas [0.67% (w/v) yeast nitrogen base without amino acids (Difco), 1% (w/v) vitamin-free casamino acids, 2% (w/v) glucose]; inducing medium, YMGRCas, was as YMMCas except that glucose was replaced by 2% (w/v) galactose plus 2% (w/v) raffinose.

Template DNA used for site-directed mutagenesis was prepared from the *E.coli* strain BW313 {HfrKL16 PO/45 [*lysA* (61-62)], *dut1, ung1, thi1, relA1*; Kunkel *et al.*, 1987}. For general cloning and sequencing, the strain NM522 [Δ(lac, pro), hsdΔ5, supE, thi$^-$, F(*lacI^q*, *lacZ*ΔM15, pro$^+$); Messing *et al.*, 1977] was used.

Plasmid pFL45-PRP2 was constructed by cloning the 3.2 kb *Eco*RI−*Bam*HI fragment encoding *PRP2* from pY2016 (Lee *et al.*, 1984) into the polylinker of pFL45 (2μ, *TRP1*; Bonneaud *et al.*, 1991). Plasmid pBM-PRP2 (*ARS, CEN, URA3*), containing the *PRP2* gene fused to the *GAL1* promoter was described previously (King and Beggs, 1990).

Generating and sequencing dominant negative mutants

Plasmid pBM-PRP2 was subjected to hydroxylamine mutagenesis as described by Rose *et al.* (1990) and introduced directly into the yeast strain S150-2B. Following localization of the *PRP2-dn1* mutation to a central *Hin*dIII−*Xba*I region of the gene (see Results), this DNA fragment was subcloned into pTZ18R (Invitrogen) and the entire 777 bp region was sequenced. To confirm that the single transition mutation revealed by sequencing the *Hin*dIII−*Xba*I region of the *PRP2-dn1*, *PRP2-dn2* and *PRP2-dn3* alleles was the cause of the dominant inhibitory phenotype, site-directed mutagenesis was carried out (Kunkel *et al.*, 1987), using the mutant oligonucleotide 5′-CATTGTTGCTAATGATATAAGC-3′ (OSWEL DNA Service, Edinburgh) to introduce an identical mutation into the *Hin*dIII−*Xba*I region of *PRP2*. The mutagenized *PRP2* fragment was used to replace the corresponding region of pBM-PRP2.

Nucleic acids methods

For Northern blot analysis, yeast total RNA was prepared by the method of Hopper *et al.* (1978), followed by denaturing agarose gel electrophoresis (Jackson *et al.*, 1988). RNA was transferred to Hybond-N membrane as recommended by the manufacturer (Amersham). DNA fragments of cloned genes were radiolabelled by the random priming method of Feinberg and Vogelstein (1984). Prehybridization and hybridization were carried out at 42°C in 0.2% (w/v) each of BSA, polyvinyl pyrrolidone and Ficoll-400, 50 mM Tris−HCl pH 7.5, 0.1% (w/v) sodium pyrophosphate, 1% (w/v) SDS, 50% (v/v) formamide, 1 M NaCl and 10 μg/ml denatured sonicated salmon sperm DNA (Stratagene); washing was 2×10 min at 42°C in 2×SSC, 2×30 min at 65°C in 2×SSC plus 0.5% (w/v) SDS and 2×30 min at room temperature in 0.1×SSC.

To produce RNA substrates for *in vitro* splicing reactions, pT7rp28 DNA (Whittaker and Beggs, 1991) was linearized by cleavage with *Eco*RI and transcribed with T7 RNA polymerase *in vitro*.

Splicing extract preparation and in vitro splicing reactions

Yeast whole cell extracts were prepared as described by Lin *et al.* (1985). Heat inactivation of extracts prepared from the *prp2-1* mutant strain DJY85 was accomplished *in vitro* by incubation at 32°C under mock splicing conditions (without pre-mRNA and ATP) for ~40 min. Heat-treated extracts regained splicing competence upon addition of purified PRP2 protein. *In vitro* splicing reactions were performed as described by Lin *et al.* (1985). To determine the minimum volume of PRP2^{dn1}-containing extract required to inhibit splicing in a wild-type extract, combinations of appropriate dilutions of extracts were mixed and pre-incubated for 10 min at 25°C prior to assembling splicing reactions. Reaction products were fractionated on 6% (w/v) polyacrylamide−8 M urea gels and visualized by autoradiography. Native gel electrophoresis was carried out according to Pikielny *et al.* (1986), but with 10 mM EDTA in the gel and running buffer. Immunoprecipitations were carried out using 10 μl splicing reactions as described by King and Beggs (1990).

Purification of polyhistidine-tagged PRP2 protein

The plasmids pBM-PRP2 and pBM-PRP2^{dn1} were modified to encode an additional 15 amino acids, including six contiguous histidine residues, at the extreme N-terminus of the PRP2 protein. The sequence CATATTATG(CAT)$_6$GGTACCATCGAGGGCCGACTC was inserted at the *Bam*HI site between the *GAL1* promoter and the *PRP2* coding sequence. Polyhistidine-tagged PRP2 protein was purified in a two-step procedure from yeast strain S150-2B harbouring pBM-PRP2 or pBM-PRP2^{dn1}. Cells were grown under inducing conditions for 6 h and splicing extracts were prepared according to Lin *et al.* (1985) except that DTT was omitted from all buffers (all procedures carried out at 4°C).

The first purification step involved metal affinity chromatography (modified from Hoffmann and Roeder, 1991). A nickel-chelating column (Invitrogen) was equilibrated with 20 mM Tris−HCl pH 7.9, 0.5 M NaCl. An equal volume of 2×sample buffer [20% (v/v) glycerol, 20 mM Tris−HCl pH 7.9, 1 M NaCl] and imidazole/HCl pH 7.9 to a final concentration of 1 mM were added to yeast cell extract before loading onto the nickel column. The extract was circulated and the column was washed with 30 ml BC100 [20% (v/v) glycerol, 20 mM Tris−HCl pH 7.9, 100 mM KCl] containing 20 mM imidazole−HCl pH 7.9, followed by 20 ml BC100 containing 49 mM imidazole−HCl pH 7.9. PRP2 protein was step-eluted with 5 ml BC100 containing 150 mM imidazole−HCl pH 7.9 followed by 7 ml BC100 containing 200 mM imidazole−HCl pH 7.9. Eluate fractions were collected and analysed by SDS−PAGE, followed by silver staining or Western blotting to identify the peak elution fractions.

Peak fractions from the nickel-chelating column were pooled and dialysed against 20 mM HEPES pH 7.7, 0.5 mM EDTA, 5 mM DTT, 0.01% (v/v) Nonidet P-40, 10% (v/v) glycerol and 50 mM KCl. Subsequently, PRP2 proteins were purified by poly(U)−agarose chromatography as described by Kim *et al.* (1992). Again, eluate fractions were analysed by SDS−polyacrylamide gel electrophoresis and silver staining to identify the peak PRP2 fractions.

The purified PRP2 protein was demonstrated to be functional by complementation of the splicing defect of a heat-inactivated prp2-1 extract (data not shown). The ability of purified PRP2^{dn1} protein to inhibit pre-mRNA splicing and cause the accumulation of inactive splicing complexes was assessed by adding various dilutions of PRP2^{dn1} protein to a splicing reaction containing active PRP2dn1−GLU extract (data not shown).

ATPase assays

Release of ^{32}P from [γ-^{32}P]ATP following hydrolysis by wild-type or mutant forms of PRP2 protein was measured using a method adapted from Iggo and Lane (1989). In a total volume of 20 μl, 60 ng of purified PRP2 protein were incubated for 0−90 min in the presence of 50 mM triethanolamine pH 8.2, 75 mM potassium acetate, 1.25 mM $MgCl_2$, 1 mM ATP, 1 μCi [γ-^{32}P]ATP (10 Ci/mmol) and 1.25 mM DTT. Where indicated, poly(U) RNA was included at a concentration of 0.5 mg/ml. Reactions were stopped on ice. Norit charcoal was pre-washed with 50 mM HCl−5 mM H_3PO_4 suspended at 5% (v/v) in 20 mM H_3PO_4 and added to 200 μl per reaction. Samples were incubated on ice for 10 min, spun at 12 000 *g* for 10 min to pellet adsorbed ATP and the free ^{32}P in the supernate was measured by Cerenkov counting. The background resulting from incubation in the absence of enzyme was subtracted from each value. Each data point represents the average of two independent results which did not differ by >20%.

Acknowledgements

We are extremely grateful to Stefan Teigelkamp, David Finnegan, Peter Hodges and Andy Newman for helpful discussions and critical comments on the manuscript. The anti-trimethylguanosine antibodies were a generous gift from Reinhard Lührmann. This work was supported by the Cancer Research Campaign. M.P. was a recipient of a Science and Engineering Research Council Studentship. J.D.B. holds a Royal Society E.P.A. Cephalosporin Fund Senior Research Fellowship.

References

Baldari,C., Murray,J.A.H., Ghiara,P., Cesareni,C. and Galeotti,C.L. (1987) *EMBO J.*, **6**, 229–234.
Bonneaud,N., Ozier-Kalogeropoulus,O., Li,G., Labouesse,M., Minivielle-Sebastia,L. and Lacroute,F. (1991) *Yeast*, **7**, 609–615.
Burgess,S., Couto,J.R. and Guthrie,C. (1990) *Cell*, **60**, 705–717.
Burgess,S.M. and Guthrie,C. (1993) *Cell*, **73**, 1377–1391.
Cech,T.R. (1985) *Cell*, **43**, 713–716.
Chen,H.-J. and Lin,R.-J. (1990) *Nucleic Acids Res.*, **18**, 6447.
Company,M., Arenas,J. and Abelson,J. (1991) *Nature*, **349**, 487–493.
Dalbadie-McFarland,G. and Abelson,J. (1990) *Proc. Natl Acad. Sci. USA*, **83**, 1271–1275.
Deschavanne,P.J. and Harosh,I. (1993) *Mol. Microbiol.*, **7**, 831–835.
Feig,L.A. and Cooper,G.M. (1988) *Mol. Cell. Biol.*, **8**, 3235–3243.
Feinberg,A.P. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266–267.
Fleig,U.N., Gould,K.L. and Nurse,P. (1992) *Mol. Cell. Biol.*, **12**, 2295–2301.
Fuller-Pace,F.V. and Lane,D.P. (1992) In Eckstein,F. and Lilley,D.M.J. (eds), *Nucleic Acids and Molecular Biology*. Springer Verlag, Berlin, Vol. 6, pp. 159–173.
Fuller-Pace,F.V., Nicol,S.M., Reid,A.D. and Lane,D.P. (1993) *EMBO J.*, **12**, 3619–3626.
Gorbalenya,A.E., Koonin,E.V., Donchenko,A.P. and Blinov,V.M. (1989) *Nucleic Acids Res.*, **17**, 4713–4730.
Green,M.R. (1991) *Annu. Rev. Cell Biol.*, **7**, 559–599.
Guthrie,C. (1991) *Science*, **253**, 157–163.
Herskowitz,I. (1987) *Nature*, **329**, 219–222.
Hirling,H., Scheffner,M., Restle,T. and Stahl,F. (1989) *Nature*, **339**, 562–564.
Hoffmann,A. and Roeder,R.G. (1991) *Nucleic Acids Res.*, **19**, 6337–6338.
Hopper,A.K., Banks,F. and Evangelidis,V. (1978) *Cell*, **14**, 211–219.
Iggo,R.D. and Lane,D.P. (1989) *EMBO J.*, **8**, 1827–1831.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Jackson,S.P., Lossky,M. and Beggs,J.D. (1988) *Mol. Cell. Biol.*, **8**, 1067–1075.
Kim,S.-H., Smith,J., Claude,A. and Lin,R.-J. (1992) *EMBO J.*, **11**, 2643–2654.
King,D.S. and Beggs,J.D. (1990) *Nucleic Acids Res.*, **18**, 6559–6564.
Konarska,M.M., Grabowski,P.J., Padgett,R.A. and Sharp,P.A. (1985) *Nature*, **313**, 552–557.
Koonin,E.V. (1991) *Nature*, **352**, 290.
Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Lain,S., Reichmann,L.J. and Garcia,J.A. (1990) *Nucleic Acids Res.*, **18**, 7003–7006.
Leberer,E., Dignard,D., Hougan,L., Thomas,D.Y. and Whiteway,M. (1992) *EMBO J.*, **11**, 4805–4813.
Lee,C.-G. and Hurwitz,G. (1993) *J. Biol. Chem.*, **268**, 16822–16830.
Lee,M.G., Young,R.A. and Beggs,J.D. (1984) *EMBO J.*, **3**, 2825–2830.
Lin,R.-J., Newman,A.J., Cheng,S.-C. and Abelson,J. (1985) *J. Biol. Chem.*, **260**, 14780–14792.
Lin,R.-J., Lustig,A.J. and Abelson,J. (1987) *Genes Dev.*, **1**, 7–18.
Linder,P., Lasko,P.F., Ashburner,M., Leroy,P., Nielsen,P.J., Nishi,K., Schnier,J. and Slonimsky,P.P. (1989) *Nature*, **337**, 121–122.
Mendenhall,M.D., Richardson,H.E. and Reed,S.I. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 4426–4430.
Messing,J., Gronenborn,B., Muller-Hill,B. and Hofschneider,P.H. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 3642–3646.
Miraglia,L., Seiwart,S., Igel,A.H. and Ares,M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7061–7065.
Moore,M.J., Query,C.C. and Sharp,P.A. (1993) In Gesteland,R.F. and Atkins,J.F. (eds), *The RNA World*. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 303–357.
Naumovski,L. and Friedberg,E.C. (1986) *Mol. Cell. Biol.*, **6**, 1218–1227.
Padgett,R.A., Konarska,M.M., Grabowski,P.J., Hardy,S.F. and Sharp,P.A. (1984) *Science*, **224**, 898–903.
Pause,A. and Sonenberg,N. (1992) *EMBO J.*, **11**, 2643–2654.
Pikielny,C.W., Rymond,B.C. and Rosbash,M. (1986) *Nature*, **324**, 341–345.
Ray,B.K., Lawson,T.G., Kramer,J.C., Cladaras,M.H., Grifo,J.A., Abramson,R.D., Merrick,W.C. and Thach,R.E. (1985) *J. Biol. Chem.*, **260**, 7651–7658.
Rose,M.D., Winston,F. and Heiter,P. (1990) *Methods in Yeast Genetics. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Rozen,F., Edery,I., Meerovitch,K., Dever,T.E., Merick,W.C. and Sonenberg,N. (1990) *Mol. Cell. Biol.*, **10**, 1134–1144.
Schmid,S.R. and Linder,P. (1992) *Mol. Microbiol.*, **6**, 283–292.
Schwer,B. and Guthrie,C. (1991) *Nature*, **349**, 494–499.
Schwer,B. and Guthrie,C. (1992) *Mol. Cell. Biol.*, **12**, 3540–3547.
Sharp,P.A. (1984) *Science*, **225**, 898–903.
Sharp,P.A. (1985) *Cell*, **42**, 397–400.
Strauss,E.J. and Guthrie.,C. (1991) *Genes Dev.*, **5**, 629–645.
Teigelkamp,S., McGarvey,M., Plumpton,M. and Beggs,J.D. (1994) *EMBO J.*, **13**, 888–897.
Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) *EMBO J.*, **1**, 945–951.
Wassarman,D.A. and Steitz,J.A. (1991) *Nature*, **349**, 463–464.
Whittaker,E. and Beggs,J.D. (1991) *Nucleic Acids Res.*, **19**, 5483–5489.

Received on September 27, 1993; revised on November 15, 1993