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Genetic interactions of conserved regions in the DEAD-box protein Prp28p

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ABSTRACT

The yeast *PRP28* gene has been implicated in nuclear precursor messenger RNA (pre-mRNA) splicing, a two-step reaction involved in a multitude of RNA structural alterations. Prp28p, the gene product of *PRP28*, is a member of the evolutionarily conserved DEAD-box proteins (DBPs). Members of DBPs are involved in a variety of RNA-related biochemical processes, presumably by their putative RNA helicase activities. Prp28p has been speculated to play a role in melting the duplex between U4 and U6 small nuclear RNAs (snRNAs), leading to the formation of an active spliceosome. To study the function of Prp28p and its interactions with other components of the splicing machinery, we have isolated and characterized a large number of *prp28* conditional mutants. Strikingly, many of these *prp28* mutations are localized in the highly conserved motifs found in all the DBPs. Intragenic reversion analysis suggests that regions of motifs II, III and V, as well as of motifs I and IV, in Prp28p are likely to be in close proximity to each other. Our results thus provide the first hint of the local structural arrangement for Prp28p, and perhaps for other DBPs as well.

INTRODUCTION

The removal of introns from nuclear pre-mRNAs, referred to as pre-mRNA splicing, proceeds via two consecutive transesterification reactions (1). In the first step, the 5′ splice site is cleaved, yielding a 5′ exon and a lariat intron–3′ exon as splicing intermediates. This is followed by cleavage at the 3′ splice site and ligation of the two exons, with the release of the intron in a lariat form. This entire splicing process is carried out in the spliceosome, a macromolecular complex consisting of a large number of proteins and several small nuclear RNAs (snRNAs). Five spliceosomal snRNAs, U1, U2, U4, U5 and U6, organized in the form of small nuclear ribonucleoprotein particles (snRNPs), are essential for splicing. Among the five snRNAs, U6 is the most conserved and was thought to be central to the splicing catalytic activities (2,3). Since U6 and U4 snRNAs are stably base-paired with each other in a U4/6 snRNP (2,4), it has been suggested that U4 could act as an antisense RNA to regulate the proposed catalytic function of the U6 snRNA (5).

Pre-mRNA splicing *in vitro* proceeds by a sequential assembly of U1, U2 and a U4/U5/U6 tri-snRNP onto the pre-mRNA substrate, leading to the formation of a pre-splicing complex. The subsequent formation of the active spliceosome complex requires the dissociation of U4 from the pre-splicing complex (6–8), which occurs either concomitantly to or after the first cleavage at the 5′ splice site (8). It has been speculated that this step is energy-dependent and that ATP is required for RNA helicases to unwind the U4/U6 duplex, which exhibits a melting temperature (T_m) of 53°C (2). Similar RNA unwinding activities may also be needed to disrupt the U1/pre-mRNA duplex formed during spliceosome assembly.

Mammalian eukaryotic translation initiation factor eIF4A (9) and human p68 (10) were among the first proteins shown to use ATP hydrolysis to drive the unwinding of the double-stranded RNA substrates *in vitro*. It is now clear that eIF4A and p68 belong to a growing protein family called the DEAD-box protein (DBP) family, so named because of the presence of the highly conserved Asp-Glu-Ala-Asp (DEAD) motif (11,12). Members of this family are similar to each other in sequence and share more than seven highly conserved motifs with eIF4A and p68 (12). Thus, they are all thought to be RNA helicases which can promote separation of strands in RNA duplexes or removal of secondary structures in single-stranded RNA. These putative RNA helicases are found to be involved in a variety of biological processes (13), such as translation initiation, ribosomal biogenesis, cell growth and division regulation, *Drosophila* oocyte formation and specification of embryonic posterior structures and mouse spermatogenesis. The complexity and the importance of these DBPs is perhaps most obvious in yeast, where more than 26 different *DBP* genes have been identified (14) and at least 16 of these are essential for cell viability (T.-H.Chang, unpublished). There are other families of proteins which are closely related to the DBP family; these are sometimes called the DEAH and the DEXH families which, together with the DBP family, form the helicase superfamily II (11). Members of the DEAH and the DEXH families are involved in DNA replication and recombination and include putative RNA helicases of several positive-strand RNA viruses.

To investigate the role of the potential RNA unwinding activities in mRNA splicing, we have previously isolated five new yeast *DBP* genes (15). One of them, *CA8* (15), was shown to be identical to *PRP28* (16). In yeast, more than 30 *PRP* (*P*re-*R*NA *P*rocessing) genes involved in nuclear pre-mRNA processing have been identified by various genetic means (1). Five PRP proteins are members of the helicase superfamily II. Two of these, Prp28p (16) and Prp5p (17) are members of the DBP family. The other three, Prp2p (18), Prp16p (19) and Prp22p (20), belong to the DEAH family. Of these five proteins, Prp2p, Prp5p, Prp16p and Prp28p

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have been shown to possess the predicted RNA-dependent ATPase activities. However, evidence for their RNA unwinding activities is lacking. It has been reported that the mammalian eIF4A requires a cofactor, eIF4B, for its optimal RNA helicase activity *in vitro* (9). By analogy, it is possible that other DBPs, such as Prp28p, may also require auxiliary factors for their unwinding activities.

We are interested in studying the functions of Prp28p in pre-mRNA splicing. To facilitate a comprehensive genetic and biochemical analysis of Prp28p, we sought to isolate a large number of *prp28* conditional mutants. We found that many of these mutations are located in the conserved DBP motifs, suggesting a probable and convenient way for constructing analogous conditional mutants in other *DBP* genes. Intragenic reversion analysis of these *prp28* conditional mutants has permitted us to propose the first structural arrangement among four of the highly conserved motifs in Prp28p.

MATERIALS AND METHODS

Cloning and sequencing of *PRP28*

A YCp50 genomic library was screened by bacterial colony hybridization for clones carrying the *PRP28* gene using a [^{32}P]dCTP-labeled probe, *CA8* (15). On the basis of the Southern analysis, a DNA fragment thought to encompass the entire *PRP28* gene was subcloned into Bluescript KS(+) and KS(–) vectors (Stratagene, La Jolla, CA) in both orientations. Two sets of nested deletion clones, one from each orientation, were constructed using an *Exo*III/Mung Bean Deletion Kit from Stratagene. Single-stranded DNA templates were prepared from each individual clone for dideoxy chain termination sequencing with a Sequenase Kit (U.S. Biochemical Corp., OH). A total of 4451 nucleotides were determined. Sequence of the *PRP28* gene (2452 nucleotides in total) was independently determined by Strauss and Guthrie (16). Our sequence extends 1980 nucleotides beyond the 3′-end of the published sequence.

Plasmids

Plasmids used in this study are summarized in Table 1. Three centromere yeast shuttle vectors (21), pRS314 (*TRP1*), pRS315 (*LEU2*) and pRS316 (*URA3*) were used in this study.

Yeast strains

A *Spe*I–*Eco*RI fragment containing the *prp28::HIS3* allele was isolated from pCA8033 (Table 1) and transformed into a diploid yeast strain YPH274 (*MAT***a***/MAT*α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-*Δ*1/trp1-*Δ*1 his3-*Δ*200/his3-*Δ*200 leu2-*Δ*1/leu2-*Δ*1*; 21). Yeast genomic DNAs isolated from the His$^+$ transformants were used to screen by Southern analysis for transformants in which one of the two *PRP28* genes was replaced by the *prp28::HIS3* allele. The confirmed diploid strain, YTC59, has the following genotypes: *MAT***a***/MAT*α *prp28::HIS3/PRP28 ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-*Δ*1/trp1-*Δ*1 his3-*Δ*200/his3-*Δ*200 leu2-*Δ*1/leu2-*Δ*1*. Transformation of strain YTC59 by pCA8032 (Table 1) yielded strain YTC63. Strain YTC65 [*MAT*α *ura3-52 lys2-801 ade2-101 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ*1 prp28::HIS3* (*pCA8032*)] was derived from sporulation of YTC63.

Oligonucleotide-directed site-specific mutagenesis

Fifteen oligonucleotides (sequences available upon request) were designed to alter the highly conserved amino acids in Prp28p. Single-stranded DNA derived from plasmid pCA8005 (Table 1) was used as template for site-specific mutagenesis which was done as described (22) using reagents from Bio-Rad Laboratories (Hercules, CA). Positive clones were verified by DNA sequencing. Mutant DNA inserts were then recloned into pRS314 for plasmid shuffling (see below).

Hydroxylamine mutagenesis

Plasmid pCA8034 (*LEU2*) was reacted with hydroxylamine to yield 2.6% leucine auxotrophy as described (23). After transformation of the mutagenized DNA into *Escherichia coli*, ~24 000 transformants were pooled for preparing a mutant *prp28* library termed pCA8034-[mut].

PCR mutagenesis

A 950 bp DNA fragment was PCR-amplified by primers 28-19 (agtactagatgaagctga) and 28-18 (aagataaaaggatccaaacatatt) using plasmid pCA8009 (Table 1) as template. Primer 28-19 is complementary to the VLDEAD region (motif II) in *PRP28* and primer 28-18, which contains an engineered *Bam*HI site (underlined), is complementary to a region ~190 bp downstream from the stop codon. Mutagenic conditions described by Cadwell and Joyce (24) were used for PCR amplification. The resulting product was isolated and re-amplified under non-mutagenic conditions. The final PCR product was digested with *Bam*HI and *Mun*I, which cuts 5′ to motif IV (IIF). The resulting 650 bp *Mun*I–*Bam*HI fragment, covering a region from motif IV to motif VI, was used to replace a cognate fragment in plasmid pCA8056 (Table 1). Approximately 6000 bacterial transformants were pooled for preparing a second mutant *prp28* library termed pCA8056-[mut].

Table 1. Plasmids used in this study

Plasmid	Description
pCA8005	A 3.6 kb *Kpn*I fragment containing *PRP28* was cloned into Bluescript KS(+) vector for site-specific mutagenesis
pCA8009	A 3.6 kb *Kpn*I fragment containing *PRP28* was cloned into pRS316 vector (*CEN/URA3*) for PCR mutagenesis
pCA8031	A 1.5 kb *Bcl*I–*Bam*HI fragment downstream of *PRP28* was deleted from pCA8005 for constructing the *prp28::HIS3* allele (see pCA8033)
pCA8032	A 2.1 kb *Kpn*I–*Bcl*I fragment containing *PRP28* was cloned into pRS316 vector (*CEN/URA3*)
pCA8033	An internal 1.7 kb *Bgl*II fragment of *PRP28* in pCA8031 was replaced by *HIS3* carried on a 1.7 kb *Bam*HI fragment to yield the *prp28::HIS3* allele
pCA8034	A 2.1 kb *Kpn*I–*Bcl*I fragment containing *PRP28* was cloned into pRS315 vector (*CEN/LEU2*) for hydroxylamine mutagenesis
pCA8056	A 2.1 kb *Kpn*I–*Bcl*I fragment containing *PRP28* was cloned into pRS314 vector (*CEN/TRP1*) for PCR and site-specific mutagenesis

Plasmid shuffling and screening of conditional mutants

Site-specific mutagenized plasmids, pCA8034-[mut] library, and pCA8056-[mut] library were transformed into yeast strain YTC65 (see above). Selection for pCA8032 (*URA3*), which carries the wild-type *PRP28* gene, in YTC65 was omitted, thus allowing this plasmid to be lost freely. For each site-specific mutant, four independent transformants were streaked out on 5-fluoroorotic acid (5-FOA) plates at 30°C to counterselect cells in which pCA8032 has not been lost. The isolated single colonies were then analyzed. The inability to isolate viable single colonies on 5-FOA plates at 30°C was taken as an indication that the engineered mutation was lethal.

For hydroxylamine and PCR mutagenesis, transformants were replica-plated onto four 5-FOA plates which were then incubated at 30, 37, 25 and 15°C, respectively. Conditional mutants were picked from the master plates and re-streaked on 5-FOA plates for isolation of single colonies. To confirm the growth phenotypes of the isolated mutants, mutagenized plasmids were recovered and reintroduced into YTC65 for a second round of 5-FOA counterselection. Only plasmid-linked mutations were further analyzed. Mutations were determined by sequencing the entire coding region of *PRP28*. A total of nine and seven conditional mutants were isolated by screening 30 000 and 14 000 transformants from hydroxylamine and PCR mutagenesis experiments, respectively (Table 2).

Table 2. Summary of *prp28* mutants

Mutation	Location	Phenotype	Allele name	Methodology
A221V	motif I	Ts^- + Cs^-	*prp28-103*	O[a]
T223I	motif I	no growth at 25ºC or lower	*prp28-117*	H[b]
R264E	motif Ia	lethal		O
R264D	motif Ia	lethal		O
E265Q	motif Ia	Ts^- + Cs^-	*prp28-99*	O
Q268/UAG	motif Ia	Ts^- + Cs^-	*prp28-76*	H
T317I	motif Ib	no growth at 37, 25 and 37°C	*prp28-36*	H
T317Y	motif Ib	lethal		O
G319V	motif Ib	Ts^- + Cs^-	*prp28-100*	O
G319E	motif Ib	Ts^- + Cs^-	*prp28-17*	H
M376I	motif III	Cs^-	*prp28-32*	H
A379W	motif III	no growth at 25ºC or lower	*prp28-102*	O
A379V	motif III	wild-type		O
F442H	motif IV	wild-type		O
F442G	motif IV	Ts^- + Cs^-	*prp28-101*	O
F442S	motif IV	Ts^-	*prp28-55*	P[c]
M491K	conserved	Ts^-	*prp28-52*	P
R499K	motif V	Cs^-	*prp28-86*	H
R499G	motif Vl	lethal		O
R499E	motif V	wild-type		O
D502N	motif V	lethal		O
Y521D	motif VI	Ts^-	*prp28-37*	P
R527K	motif VI	wild-type		O
R527D	motif VI	lethal		O
Q56/UAA	–	Ts^- + Cs^-	*prp28-59*	H
T274I + Q275/UAA	–	Ts^-	*prp28-98*	H
Q481/UAA	–	Ts^-	*prp28-47*	H
L480/UAG	–	Ts^-	*prp28-46*	P
I440F + F546S + N584E	–	Ts^-	*prp28-56*	P
P438L + H468L + N486D	–	Ts^-	*prp28-61*	P
A449T + V541A + L549V + K580N + I586V	–	Ts^-	*prp28-66*	P

[a]Oligonucleotide-directed site-specific mutagenesis.
[b]Hydroxylamine mutagenesis.
[c]PCR mutagenesis.

Isolation and characterization of intragenic suppressors

Four conditional *prp28* strains (see Results) were used for isolation of revertants capable of growing at the non-permissive temperatures. Cells were grown to saturation, diluted and plated on YPD (1% yeast extract, 2% peptone, 2% dextrose) for isolation of spontaneous revertants as described (25). Plasmids carrying the original *prp28* mutation from each revertant were recovered and re-introduced into strain YTC65. The growth phenotypes of the resulting strains after 5-FOA counterselection of pCA8032 were then examined. Plasmids which yielded strains with identical growth phenotype to the parental conditional mutants were considered to harbor only the original *prp28* mutations. Plasmids which rendered a wild-type growth phenotype were assumed to either have reverted the original *prp28* mutation or to have acquired a second-site suppressor mutation(s) within the *PRP28* gene. The locations of the intragenic suppression mutations were then determined by DNA sequencing.

Northern analysis

Northern analyses were done as described (26). Yeast cultures were grown in YPD at permissive temperature to 0.8–1.5 OD_{600} and divided into two aliquots for temperature shift experiments. One of the aliquots was shifted to 37 or 15°C for 2 h prior to extraction of the total RNAs. We used a [^{32}P]dCTP-labeled probe prepared from a 2.2 kb *Hin*dIII DNA fragment, which consists of an intron-containing gene, *CRY1*, and the *SNR189* gene (27), to examine the accumulation of the *CRY1* pre-mRNA. The *SNR189* gene does not contain intron and serves as a control for amounts of RNA loaded in each lane.

RESULTS

Cloning and sequencing of the *PRP28* gene

We have previously identified five new *DBP* genes in yeast *Saccharomyces cerevisiae* (15). One of them, originally designated as *CA8*, was cloned and sequenced and found to be identical to *PRP28*. *PRP28* is the wild-type allele of a cold-sensitive mutation which impairs nuclear pre-mRNA splicing *in vivo* (16). *PRP28* is an essential gene as shown by standard gene disruption experiments (16; data not shown). The predicted molecular mass for Prp28p is 66.6 kDa, in good agreement with the size of the overproduced Prp28p in *Escherichia coli* (T.-H.Chang, unpublished). We noted that there is a potential leucine zipper, L_{300}-6X-L_{307}-6X-L_{314}-6X-L_{321}-7X-L_{329}-L_{330} (amino acids numbered in subscripts), in the predicted Prp28p sequence.

Strategies for constructing conditional *prp28* mutants

Sequence alignments revealed that there are at least seven conserved motifs present in all the DBPs (Fig. 1) (11). Several of these conserved motifs in mammalian and yeast eIF4A proteins have been studied by site-specific mutagenesis (28–31); these include motif I (A/S-X-T/S-G-S/T-GKT), motif II (DEAD), motif III (S/T-AT) and motif VI [Y/F-(Hb)-HRIGR-T/G-R where (Hb) is a hydrophobic amino acid]. To investigate these and other conserved motifs further, we constructed a total of 16 *prp28* mutants by site-specific mutagenesis and examined their growth phenotypes using a plasmid shuffling protocol (23). We also used hydroxylamine mutagenesis to generate random mutations within the coding region of *PRP28* and a PCR approach to target mutations to the region of motif VI, which has been implicated in RNA binding for mammalian eIF4A (29). The mutagenized *PRP28* clones were then introduced into strain YTC65 for screening *prp28* mutants failed to grow at either 37 (temperature-sensitive mutants; Ts$^-$) or 15°C (cold-sensitive mutants; Cs$^-$). Remarkably, many of these *prp28* mutations were found to localize in the highly conserved regions present in all the DBPs. These results are summarized in Figure 1 and Table 2.

Mutations in motif I

Motif I (A/S_{221}-X-T/S_{223}-G-S/T-GKT), also known as ATPase A motif, is present in the majority of nucleotide-binding proteins (32,33). In most of the DBPs, the first position of this motif is alanine. Substitutions of this alanine residue with serine or glycine in mammalian eIF4A were neutral (28) and similar alanine-to-glycine change in yeast eIF4A had no effect on the yeast cell growth (31). These data were consistent with the fact that this position is occupied by a serine residue in Spb4p and Prp5p and by a glycine residue in Prp2p, Prp16p and Prp22p. However, in yeast eIF4A, an alanine-to-valine mutation is lethal (31), and the same mutation in mammalian eIF4A abolishes the ATP-binding and consequently the ATPase and the RNA unwinding activities (28). We constructed an analogous mutation, A221V, in Prp28p. Surprisingly, this mutant strain was viable but grew poorly at 37 and 15°C (Fig. 2). Thus, it appeared that this valine substitution was less disruptive to the nucleotide-binding capability of Prp28p.

Among all the DBPs, the third position in motif I is occupied by either a threonine or a serine residue. However, hydrophobic substitutions, such as valine, isoleucine and glycine, can be found in nucleotide-binding proteins (32) other than DBPs. A conditional mutation at this position was isolated by random mutagenesis. This mutant, T223I, was unable to grow at 25°C or lower (Fig. 2).

Mutations in motif Ia

The function of the motif Ia (APTR$_{264}$E$_{265}$LA) remains unknown. We have made two mutations, R264D and R264E, to replace the invariant arginine in this motif. Both mutations were lethal, suggesting that a positive charge may be important at this position. The adjacent E_{265} is also highly conserved, except that in yeast MSS116 protein, it is an aspartic acid instead. A mutation at this position, E265Q, yielded a Cs$^-$ phenotype and a slightly leaky Ts$^-$ phenotype (Fig. 2), suggesting that elongating the carbon side-chain (from E to D in MSS116) or eliminating the negative charge (from E to Q) at this position can both be tolerated to a certain extent.

Mutations in motif 'Ib'

Although highly conserved, the sequence element A/G-T_{317}PG_{319}R-[Hb] was previously undesignated. Since this element is invariably located between motifs Ia and II, we therefore termed it motif 'Ib'. Two mutations, T317Y and G319V, were introduced into this sequence element. Replacing the invariable threonine with a tyrosine (T317Y) was lethal. However, changing the conserved glycine to valine (G319V) could be tolerated. This mutant strain exhibited a slow growth phenotype at both 37 and 15°C (Fig. 2). By random mutagenesis, we have isolated two additional mutations at positions 317 and 319. The T317I mutant grew substantially slower than the wild-type strain at 30°C and failed to grow at other temperatures (Fig. 2). The G319E mutation yielded both Ts$^-$ and Cs$^-$ phenotypes, reminiscent of the G319V mutant described above. The phenotypes of the G319E mutant were

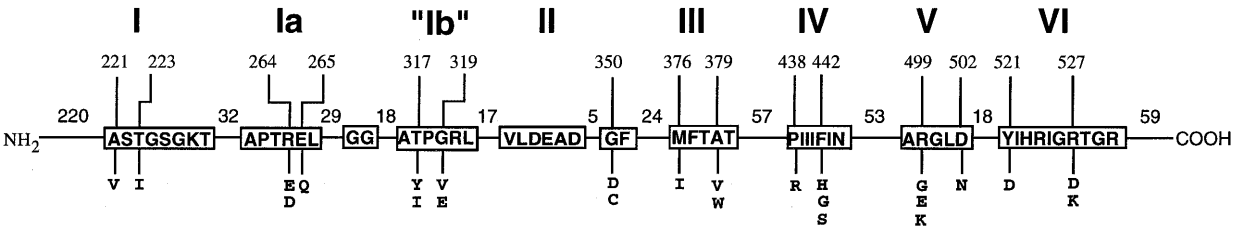

Figure 1. Schematic representation of the highly conserved sequence elements in Prp28p. The conserved regions are boxed and the numbers between the boxes indicate the distance in amino acid residues. The roman numbers above the boxes denote the nomenclature after Hodgman (42). Mutations found in the conserved regions are shown below the corresponding motifs. The numbers above the amino acids mark the positions of the amino acid residues in Prp28p. The putative leucine zipper is located from positions 300 to 330.

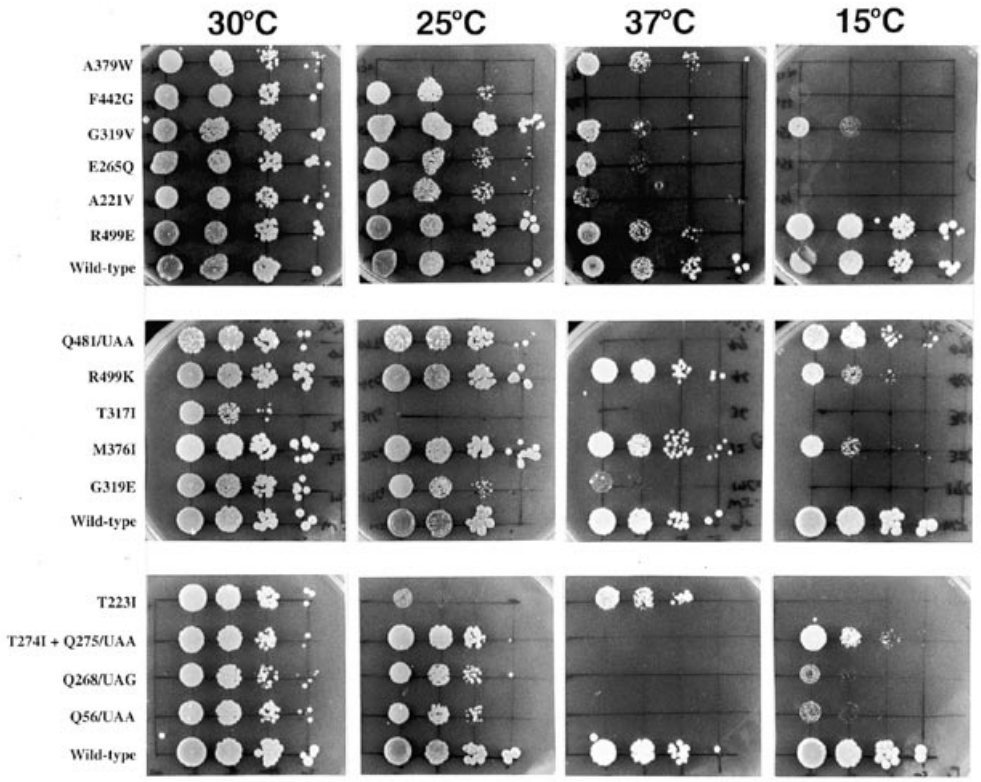

Figure 2. Growth phenotypes of the representative *prp28* mutants. Cells were grown to saturation in liquid YPD medium at 30°C, serially diluted and spotted onto YPD plates. A set of four plates were incubated at 30, 25, 37 and 15°C, respectively. Mutations of these *prp28* alleles are shown to the left.

noticeably tighter than that of the G319V mutant (Fig. 2), perhaps reflecting a more dramatic change in G319E than in G319V.

Mutations in motif III

Motif III ($LM_{376}F$-S/T-$A_{379}T$) has been studied in mammalian eIF4A. Mutations changing SAT to AAA completely eliminated the RNA helicase activity without affecting other biochemical activities (28). We have designed two mutations, A379V and A379W, to assess the importance of the invariant alanine in this motif. The A379V mutant grew normally at all temperatures tested. However, the A379W mutant failed to grow at 25 and 15°C (Fig. 2), suggesting that the sheer size of the side chain group at this position is important for Prp28p's function. In addition, our random mutagenesis yielded a mutation, M376I, within this region. This mutation gave rise to a slightly leaky Cs^- phenotype (Fig. 2). In DBPs, this position is occupied by either methionine, leucine or valine. Isoleucine at the analogous position can only be found in some DEAH-Box proteins (e.g. Prp2p and Prp16p).

Mutations in motif IV

The function of the conserved motif IV (IIF_{442}) is unknown. Here we have mutated the invariant phenylalanine to either histidine (F442H) or glycine (F442G) to assess the contributions of the side-chain groups. Surprisingly, the F442H mutant displayed no detectable growth phenotype, whereas the F442G mutant failed to grow at 37 and 15°C (Fig. 2). These results suggested that the replacement of F_{442} by another residue containing a bulky aromatic ring (i.e. F422H) could be accommodated. Depending

on its local environment, histidine can be either uncharged or positively charged. In this case, the histidine mutation was likely to be uncharged, since the original phenylalanine at this position is absolutely conserved among all the DBPs. In addition, our PCR mutagenesis also yielded a mutation, F442S, mapped to exactly the same position. This mutant exhibited a Ts$^-$ phenotype.

Mutations in motif V

The function of the motif V, A/S/I-R$_{499}$G-[Hb]-D$_{502}$, has not been characterized. Two mutations, R499G and R499E, were targeted to the conserved arginine in this motif. In addition, a R499K mutation was isolated by random mutagenesis. The R499G mutation was lethal and the R499K mutant exhibited a Cs$^-$ growth phenotype. However, the R499E mutation surprisingly yielded a neutral phenotype (Fig. 2), seemingly arguing against the absolute requirement of a positive charge at this position. Whether or not this is a unique circumstance for Prp28p remains to be investigated. The aspartic acid at position 502 is essentially invariable, except that it is asparagine in Prp5p. An analogous mutation, D502N, in Prp28p, however, yielded a lethal phenotype.

Mutations in motif VI

The motif VI, Y$_{521}$-[Hb]-HRIGR$_{527}$-T/G-R, was speculated to be involved in protein–RNA interactions, on the basis of its three conserved arginine residues (12). Schmid and Linder (31) mutated the first arginine residue in yeast eIF4A, and found that only the conservative substitution (R347K) resulted in an active protein. Sonenberg and co-workers further demonstrated that all point mutations in this region strongly reduce RNA binding activity of mammalian eIF4A and abolish its RNA helicase activity (29). We mutated the second arginine at position 527 to either an aspartic acid or lysine residue. The R527K mutant has the wild-type growth rate, whereas the R527D mutation is lethal. This result, although indicative to the importance of a positive charge at this position in Prp28p, is in sharp contrast with the mammalian eIF4A data, where an analogous R-to-K mutation (R362K) completely eliminates eIF4A's RNA helicase activity (29). In the process of generating mutations at R$_{527}$, we fortuitously isolated a lethal frame-shift mutation at this position. This single-nucleotide deletion yielded a R527E mutation and altered the downstream reading frame to produce a truncated Prp28p. By PCR mutagenesis, we have isolated one additional mutation within motif VI. Mutant Y521D failed to grow at 37°C.

Other mutations

Several conditional mutants were found to harbor either ochre (UAA) or amber (UAG) nonsense mutations in the *PRP28* gene (Fig. 2 and Table 2). These include mutations at Q$_{56}$ (CAA to UAA; phenotype: Ts$^-$ + Cs$^-$), Q$_{268}$ (CAG to UAG; Ts$^-$ + Cs$^-$), Q$_{481}$ (CAA to UAA; Ts$^-$), and a double mutant T274I/Q$_{275}$ (CAA to UAA; Ts$^-$). Among these mutations, Q$_{268}$ is highly conserved in all the DBPs. Since these mutations were predicted to be lethal, and we have ruled out the possibility that these mutations were edited at the RNA level by sequencing across the mutations on the RNA transcripts (data not shown), we suspected that these nonsense codons were decoded by some uncharacterized tRNA suppressors to yield mutant forms of Prp28p. Suppression of this sort arises often in yeast by spontaneous mutations (34).

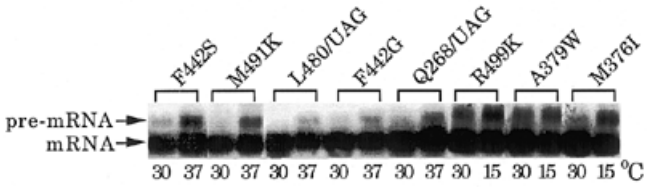

Figure 3. Northern analysis of *prp28* conditional mutants. Equal amounts of total RNA were denatured by glyoxal, fractionated on 1% agarose gel, and transferred to nylon membrane for Northern analysis. The probe detected both the pre-mRNA and the mRNA (arrows) of the *CRY1* gene. RNAs were isolated either from cells grown at 30°C or from cells which have been shifted to either 37 or 15°C for 2 h. The *prp28* mutations harbored in the strains used for these experiments were shown on the top (see also Table 2).

Our PCR mutagenesis yielded other types of mutations. Mutation M491K is in a conserved region composed of three consecutive hydrophobic amino acids (leucine, isoleucine, valine or methionine) situated seven residues upstream of the motif V (A/S/I$_{498}$-RG-[Hb]-D). Mutation at L$_{480}$ was a nonsense mutation, changing the UUG codon to UAG (amber). We have also isolated two triple and one quintuple mutants in this screen (summarized in Table 2).

Northern analysis of the *prp28* mutants

The original *prp28* cold-sensitive mutant (cs1) was found to accumulate pre-mRNAs at the non-permissive temperature (16). We thus examined the splicing defects of several of the newly isolated *prp28* mutants. As expected, these *prp28* mutants also accumulated *CRY1* pre-mRNA at the non-permissive temperatures (Fig. 3), confirming the involvement of Prp28p in pre-mRNA splicing. We noticed that some mutants accumulated small amount of *CRY1* pre-mRNA at the permissive temperature, a phenotype which has been described for several other *prp* mutants.

Intragenic suppression analysis

To investigate the structure and function relationship of Prp28p by a genetic means, we sought to isolate intragenic suppressors using four different conditional *prp28* alleles, T223I, M376I, A379W and R499K. Three spontaneous intragenic revertants were obtained from mutant strain carrying an A379W mutation in motif III (MFTA$_{379}$T). This mutant failed to grow at 25°C or lower (Fig. 2). All three revertants grew well at 15°C (data not shown). Sequencing of the recovered plasmids revealed that all three revertants possess an identical second-site mutation, G350C (Fig. 4). Remarkably, a different mutation at the same position, G350D, was found to suppress another mutation in motif V (AR$_{499}$GLD), R499K, which gave rise to a Cs$^-$ phenotype (Fig. 2). Thus, two mutations altering the conserved G$_{350}$, which is 6-residue downstream of motif II (VLDEAD), can respectively suppress two separate mutations in motifs III and V (Fig. 4). Three spontaneous intragenic revertants were also isolated from a Cs$^-$ strain harboring a mutation in the region of the motif III (M$_{376}$FTAT), M376I (Fig. 2). Sequencing analysis revealed that the second-site mutation resides at position 506, changing a conserved valine to isoleucine (V506I). V$_{506}$, which is 4-residue downstream from the ARGLD$_{502}$ motif, can probably be considered as part of the extended motif V. Taken together, these data suggest that regions of motifs II, III and V may be physically

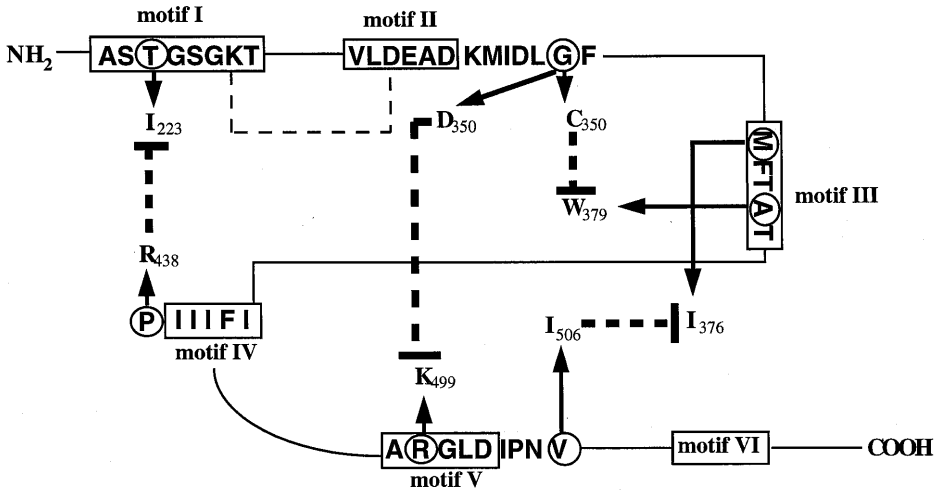

Figure 4. Summary of intragenic suppression data. Conserved regions (motifs I to VI) in Prp28p are boxed and connected by thin lines from the N-terminus (NH_2) to the C-terminus (COOH). Mutations are indicated by arrows and their positions marked with corresponding numbers. Intragenic suppressors (see text) are shown by thick dashed lines ending with short thick bars. The thin dashed line depicts the proposed close proximity between motifs I and II as determined by X-ray crystallography studies (38,39).

close to each other (Fig. 4). In another suppression analysis using a strain carrying a T223I mutation in motif I ($AST_{223}GSGKT$; Fig. 2), we recovered an intragenic revertant which acquired a second-site mutation, P438R. P_{438} is adjacent to motif IV ($I_{439}IIF$), suggesting a potential interaction between motifs I and IV.

DISCUSSION

Two *DBP* genes in yeast, *PRP5* (17) and *PRP28* (16,35), have been implicated in pre-mRNA splicing. The fact that *prp28-1* is synthetic lethal with *prp24-1*, which encodes a mutant form of Prp24p, a U6 snRNA-binding protein, and that a suppressor of *prp28-1* is a mutant allele of *PRP8*, which encodes a U5 protein, has led to a proposal that Prp28p may be involved in destablizing U4/U6 RNA duplex in the spliceosome (16). However, unlike several other DBPs which have been shown to unwind RNA duplex *in vitro* (13), the highly purified Prp28p from yeast (35) or from *E.coli* overproducing strains (T.-H.Chang, unpublished) could not unwind RNA. This was reminiscent of eIF4A whose RNA unwinding activity is strongly activated by eIF4B (9). Thus, although it is formally possible that Prp28p is not an RNA helicase, we favor the idea that other activation factors and/or specific RNA substrates may be required to trigger Prp28p's RNA unwinding activity *in vitro*. To gain insight into the requirement of the putative RNA unwinding activity for Prp28p and to elucidate its role in the yeast mRNA splicing pathway, we have undertaken an extensive search for *prp28* conditional mutants.

This search resulted in a collection of 20 conditional mutants which includes nine Ts^-, four Cs^- and seven Ts^- + Cs^- mutants (Table 2). This work rested upon an assumption that the highly conserved motifs are particularly sensitive to mutations, in as much as they are the outcome of the evolutionary conservation. This hypothesis is largely supported by our site-specific mutagenesis experiments. Among the 16 site-specific mutations we constructed, five gave rise to conditional growth phenotypes, seven were lethal and only four retained the wild-type phenotype. Furthermore, nine out of the 16 conditional mutants isolated by hydroxylamine and PCR mutagenesis contain mutations in the conserved regions (Table 2).

The biochemical functions of the conserved motifs Ia ($APTR_{264}E_{265}LA$) and 'Ib' ($A/G\text{-}T_{317}PG_{319}R\text{-}[Hb]$) are unknown. It is interesting to note that motifs Ia and 'Ib' in Prp28p appeared to be particularly susceptible to mutations. A total of seven mutations within these two motifs have been examined or isolated. Of these, three (R264D, R264E and T317Y) are lethal and four (E265Q, T317I, G319V and G319E) gave rise to Ts^- + Cs^- phenotype. Similarly, for yeast eIF4A, mutations changing the conserved glycine in motif 'Ib' can either slow down (G145S) or arrest the cell growth (G145D) (31). A small sequence element, consisting of two consecutive glycine residues, flanked by both motifs Ia and 'Ib' has been found in nearly all DBPs (Fig. 1). The original *prp28-1* allele harbors a mutation, G297D, at the second glycine residue (16). Previous studies on yeast eIF4A have shown that mutations in these two glycine residues can be either lethal (G126D) or leading to a slow growth phenotype (G127D) (31). No mutations of these two conserved glycine residues were uncovered in our search.

The motif VI (Y/F-[Hb]-HRIGR-T/G-R) is one of the most conserved regions in DBPs. Mutational analysis in yeast eIF4A indicated that most mutations made in this motif were lethal (31). Biochemical analysis showed that motif VI is required for RNA binding and ATP hydrolysis in mammalian eIF4A (29) but is not required for RNA binding in NPH-II (36). It has been speculated that the three arginine residues may directly interact with the phosphate backbone of the RNA and that this type of interaction does not work well by lysine residues, a scenario analogous to the action of the arginine-rich motif in the human immunodeficiency virus (HIV) Tat protein (37). However, mutations which change the first arginine residue in yeast eIF4A (R347K) (31) and the second arginine residue in Prp28p (R527K) (Table 2) appeared to be neutral, suggesting that the mode of the RNA-binding by motif VI may be different from that of the arginine-rich motif in Tat protein. Characterization of the RNA-binding activity of these two yeast mutant proteins should provide further insight into this issue.

Several lines of evidence suggest that eIF4A possesses multiple domains. First, eIF4A can be crosslinked simultaneously to ATP

and RNA (28). Second, ATPase and RNA helicase activities can be uncoupled by mutations in motifs II and III (28). Third, all four regions, motifs I, II, III and VI, are involved in ATP binding or ATP hydrolysis and consequently also affect RNA helicase activity (28,29). It was proposed that eIF4A first binds to ATP via a weak interaction, and the subsequent RNA binding engenders a conformational change in the ATP-binding site, resulting in tighter binding of ATP. The subsequent ATP hydrolysis and an increase in eIF4A affinity for RNA are then linked to unwinding of duplex RNA in the presence of eIF4B. We suggest that the conformational changes predicted in this kinetic model (29) may very well be facilitated by the direct interactions of various functional domains, as suggested by our intragenic suppression data on Prp28p. While it is not absolutely necessary for an intragenic second-site suppressor to be close to the mutation it suppresses, as the secondary mutations could alter the overall protein structure and result in suppression of the first mutation, the unusual 'triangular' suppression relationship among the regions of motifs II, III and V in Prp28p has led us to speculate that these three regions are physically close to each other (Fig. 4). Interestingly, X-ray crytallography studies of other nucleotide-binding proteins (38,39) showed that the first aspartate residue in the DEAD region [motif II; a variant of the Walker ATPase B motif (12)] is in close proximity to the ATPase A motif (motif I). Our suppression data further suggests that motif IV is also in the vicinity of motif I in Prp28p. Taken together, it is tempting to speculate that Prp28p, and perhaps other DBPs as well, is folded in such a compact manner through which the local conformational changes can be rapidly propagated to other functional domains by virtue of their close proximities. Limited proteolysis and X-ray crystallographic analysis should provide more insight into the structure and function of these enzymes.

There are at least 26 *DBP* genes in yeast *S.cerevisiae* (14). This multiplicity of *DBP* genes appears to hold true in other biological systems. Remarkably, at least in yeast, the majority of *DBP* genes are essential, suggesting that they are functionally distinct. It is conceivable that these putative RNA helicases achieve their unique biological roles by interacting with other cellular components and/or by acting specifically on their own RNA substrates. For example, *E.coli* DbpA can hydrolyze ATP only in the presence of the bacterial 23S rRNA (40). The information on the RNA substrates is undoubtedly crucial to the understanding of each DBP's cellular function. In this regard, the large number of *prp28* conditional mutants now provides a powerful tool to characterize Prp28p's role in yeast mRNA splicing pathway through a concerted biochemical and genetic approach. Characterization of the extragenic and the high-copy suppressors is expected to yield insight into Prp28p's interacting components and its RNA substrate. Several mutations in the highly conserved regions of mammalian eIF4A exhibit a dominant negative effect on translation *in vitro* (29). A mutation in the conserved S/T-AT motif in splicing factor Prp2p is also dominant negative (41). Systematic screening of such dominant negative *PRP28* mutants by employing the available *prp28* mutants is underway. Finally, we envisage that conditional mutants for other DBPs may be fashioned after these *prp28* mutants reported in this work.

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REFERENCES

1 Moore,M.J., Query,C.C. and Sharp,P.A. (1993) In Gesteland,R.F. and Atkins,J.A. (eds), *The RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 303–357.
2 Brow,D.A. and Guthrie,C. (1988) *Nature*, **334**, 213–218.
3 Fabrizio,P. and Abelson,J. (1990) *Science*, **250**, 404–409.
4 Hashimoto,C. and Steitz,J.A. (1984) *Nucleic Acids Res.*, **12**, 3283–3293.
5 Guthrie,C. and Patterson,B. (1988) *Annu. Rev. Genet.*, **23**, 387–419.
6 Cheng,S.-C. and Abelson,J. (1987) *Genes Dev.*, **1**, 1014–1027.
7 Lamond,A.I., Kornarska,M.M., Grabowski,P.J. and Sharp,P.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 411–415.
8 Yean,S.-L. and Lin,R.-J. (1991) *Mol. Cell. Biol.*, **11**, 5571–5577.
9 Rozen,F., Edery,I., Meerovitch,K., Dever,T.E., Merrick,W.C. and Sonenberg,N. (1990) *Mol. Cell. Biol.*, **10**, 1134–1144.
10 Hirling,H., Scheffner,M., Restle,T. and Stahl,H. (1989) *Nature*, **339**, 562–564.
11 Gorbalenya,A.E. and Koonin,E.V. (1993) *Curr. Opin. Struct. Biol.*, **3**, 419–429.
12 Linder,P., Lasko,P., Ashburner,M., Leroy,P., Nielsen, P., Nishi,K., Schnier,J. and Slonimski,P.P. (1989) *Nature*, **337**, 121–122.
13 Fuller-Pace,F.V. (1994) *Trends Cell Biol.*, **4**, 217–274.
14 Mewes,H.W., Albermann,K., Bähr,M., Frishman,D., Gleissner,A., Hani,J., Heumann,K., Kleine,K., Maierl,A., Oliver,S.G., *et al.* (1997) *Nature*, **387**, 7–65.
15 Chang,T.-H., Arenas,J. and Abelson,J. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1571–1575.
16 Strauss,E.J. and Guthrie,C. (1991) *Genes Dev.*, **5**, 629–641.
17 Dalbadie-McFarland,G. and Abelson,A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4236–4240.
18 Chen,J.-W. and Lin,R.-J. (1990) *Nucleic Acids Res.*, **18**, 6447.
19 Burgess,S., Couto,J.R. and Guthrie,C. (1990) *Cell*, **60**, 705–717.
20 Company,M., Arenas,J. and Abelson,J. (1991) *Nature*, **349**, 487–493.
21 Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
22 Kunkel,T.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.
23 Sikorski,R.S. and Boeke,J.D. (1991) *Methods Enzymol.*, **194**, 302–318.
24 Cadwell,R.C. and Joyce,G.F. (1992) *PCR Methods Appl.*, **2**, 28–33.
25 Moir,D., Stewart,S.E., Osmond,B.C. and Botstein,D. (1982) *Genetics*, **100**, 547–563.
26 Vijayraghavan,U., Company,M. and Abelson,J. (1989) *Genes Dev.*, **3**, 1206–1216.
27 Maddock,J.R., Weidenhammer,E.M., Adams,C.C., Lunz,R.L. and Woolford,J.L.Jr., (1994) *Genetics*, **136**, 833–847.
28 Pause,A. and Sonenberg,N. (1992) *EMBO J.*, **11**, 2643–2654.
29 Pause,A., Méthot,N. and Sonenberg,N. (1993) *Mol. Cell. Biol.*, **13**, 6789–6798.
30 Pause,A., Méthot,N., Svitkin,Y., Merrick,W.C. and Sonenberg,N. (1994) *EMBO J.*, **13**, 1205–1215.
31 Schmid,S.R. and Linder,P. (1991) *Mol. Cell. Biol.*, **11**, 3463–3471.
32 Saraste,M., Sibbald,P.R. and Wittinghofer,A. (1990) *Trends Biochem. Sci.*, **15**, 430–434.
33 Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) *EMBO J.*, **1**, 945–951.
34 Sherman,F. (1982) In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds) *The Molecular Biology of the Yeast Sacchromyces – Metabolism and Gene Expression*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 463–486.
35 Strauss,E.J. and Guthrie,C. (1994) *Nucleic Acids Res.*, **22**, 3187–3193.
36 Gross,C.H. and Shulman,S. (1996) *J. Virol.*, **70**, 1706–1713.
37 Calnan,B.J., Tidor,B., Biancalana,S., Hudson,D. and Frankel,A.D. (1991) *Science*, **252**, 1167–1171.
38 Pai,E.F., Krengel,U., Petsko,G.A., Goody,R.S., Kabsch,W. and Wittinghofer,A. (1990) *EMBO J.*, **9**, 2351–2359.
39 Story,R.M. and Steitz,T.A. (1992) *Nature*, **355**, 374–376.
40 Fuller-Pace,F.V., Nicol,S.M., Reid,A.D. and Lane,D.P. (1993) *EMBO J.*, **12**, 3619–3626.
41 Plumpton,M., McGarvey,M. and Beggs,J.D. (1994) *EMBO J.*, **13**, 879–887.
42 Hodgman,T.C. (1988) *Nature*, **333**, 22–23.