Nucleic Acids Research, 1994, Vol. 22, No. 25 5555–5564

The budding yeast U5 snRNP Prp8 is a highly conserved protein which links RNA splicing with cell cycle progression

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Received October 14, 1994; Revised and Accepted November 18, 1994 GenBank accession no. L29421

ABSTRACT

The *dbf3* mutation was originally obtained in a screen for DNA synthesis mutants with a cell cycle phenotype in the budding yeast *Saccharomyces cerevisiae*. We have now isolated the *DBF3* gene and found it to be an essential gene with an ORF of 7239 nucleotides, potentially encoding a large protein of 268 kDa. We also obtained an allele-specific high copy number suppressor of the *dbf3-1* allele, encoded by the known *SSB1* gene, a member of the Hsp70 family of heat shock proteins. The sequence of the Dbf3 protein is 58% identical over 2300 amino acid residues to a predicted protein from *Caenorhabditis elegans*. Furthermore, partial sequences with 61% amino acid sequence identity were deduced from two files of human cDNA in the EST nucleotide database so that Dbf3 is a highly conserved protein. The nucleotide sequence of *DBF3* turned out to be identical to the yeast gene *PRP8*, which encodes a U5 snRNP required for pre-mRNA splicing. This surprising result led us to further characterise the phenotype of *dbf3* which confirmed its role in the cell cycle and showed it to function early, around the time of S phase. This data suggests a hitherto unexpected link between pre-mRNA splicing and the cell cycle.

INTRODUCTION

The removal of introns from eukaryotic messenger RNA precursors requires two sequential transesterification reactions which take place within the complex ribonucleoprotein particle, the spliceosome. Assembly of the spliceosome involves interactions between a large number of protein factors and five small nuclear RNA molecules the U1, U2, U4, U5 and U6 snRNAs. Some of the protein factors are bound to snRNAs to form small ribonucleoproteins (snRNPs), whilst other non-snRNP proteins have only a transient association with the spliceosome. The U1, U2, U4/U6 and U5 snRNPS have been extensively studied, the U1 snRNP is the first particle to interact with the substrate mRNA precursor, this is then followed by the U2 snRNP and together they form a pre-spliceosome complex. Concurrently, the U4/U6 and U5 snRNPs associate to form a triple snRNP and this then joins with the pre-spliceosome complex to form the spliceosome [For reviews see (1–5)].

Studies in budding yeast have been useful in identifying some of the protein components of the spliceosome and include the important *prp* mutants (precursor mRNA processing) many of which were originally designated as *rna* mutants. These mutants have defects in pre-mRNA splicing which cause a temperature sensitive (ts) conditional lethal phenotype (2,6,7). Over 30 *PRP* genes have been identified to date, of which the *PRP3, PRP4, PRP5, PRP7, PRP8* and *PRP11* have been shown to be vital for spliceosome assembly *in vitro* (1,8,9).

The *PRP8* gene, the subject of this study, encodes a protein component of the U5 snRNP. The Prp8 protein is associated with the spliceosome during both transesterification reactions (10,11). Functional Prp8 protein is required for the formation of the U4/U6.U5 tri-snRNP without which the spliceosome cannot form (9). Additionally, immunological and UV crosslinking studies have shown that Prp8 interacts directly with the pre-mRNA substrate and may possess an important structural and/or regulatory role in the yeast spliceosome (12). Moreover, proteins related to Prp8 have been identified immunologically in mammalian cells and other eukaryotes which emphasises that Prp8 plays an important role in splicing since it seems to be conserved across species boundaries (11,13–15).

Analysis in yeast has demonstrated genetic interactions between *PRP8* and a number of other genes. For example, a suppressor of *prp8-1* turned out to be a dominant mutation in the *SRN1* (suppressor of RNA mutations) gene. The *SRN-1* mutation was first found to suppress the ts defect of *prp2 prp6* cells but can also suppress *rna2/prp2, rna3, rna4, rna5, rna6/prp6* and *rna8/prp8* ts mutations either singly or in pairs (16). Surprisingly,

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the *SRN1* gene was found to be the same as the *HEX2/REG1* gene which acts as a negative regulator of glucose repression (17). This finding suggests that there is a link, be it direct or indirect, between carbon catabolite metabolism and mRNA precursor splicing. In this paper we describe another unexpected link involving mRNA splicing, namely a connection with the cell cycle. We isolated the *DBF3* gene, one mutant allele of which possesses a cell cycle phenotype indicative of a defect in DNA synthesis (18,19) and were surprised to find it identical to the *PRP8* gene.

In budding yeast, genetic approaches to understanding how the cell cycle functions and is regulated began with the isolation of cell division cycle (*cdc*) mutants (20). Mutations in a *CDC* gene are generally temperature-sensitive (ts) and result in a defect in a particular cell cycle stage specific function at the restrictive temperature that leads to the accumulation of cells with a uniform morphology [reviewed in (21)]. The *dbf* (dumb-bell former) mutants are also cell cycle mutants originally isolated in an attempt to identify genes involved in the control of S phase. The three mutants *dbf2, 3* and *4* possess a ts defect in DNA synthesis, but have no apparent defect in RNA metabolism (18,19), although, significantly, one mutant, *dbf5*, was found to be allelic to *rna3/prp3* (18). The *DBF2* gene has been isolated and encodes a cell cycle regulated protein kinase functioning both in controlling S phase and also in mitosis (22,23). Its principal role is in a pathway bringing about the end of mitosis. The *DBF4* gene is expressed under cell cycle control and acts immediately prior to S phase where it is an essential subunit of the Cdc7 protein kinase (24). Indeed, Dbf4 may target Cdc7 to origins of replication as it binds to a subunit of the ORC protein complex that, in turn, binds to origins (25). The third mutant, *dbf3*, was originally characterised as having a defect in the initiation of DNA synthesis suggesting that the Dbf3 protein may function in the control of the G1/S phase transition (18,19). Consistent with an S phase role for Dbf3, other alleles of the mutant were also isolated in an independent screen for mutants defective in DNA synthesis (26).

Here we confirm that *DBF3* has a role in the cell cycle and the timing of its function coincides with S phase. At the restrictive temperature of 37°C *dbf3-1* cells arrest with an approximately 2C content of DNA but with an undivided nucleus. DNA replication probably narrowly fails to be completed in the majority of *dbf3-1* cells at the restrictive temperature. Sequencing showed *DBF3* to be identical to *PRP8* and predicted a protein of 2413 amino acid residues. This was found to be 58% homologous over 2300 amino acid residues to a predicted protein from the nematode worm *Caenorhabditis elegans*. Additionally, parts of a human *DBF3/PRP8* homologue were found which were 61% identical to the yeast sequence and 87% identical to the worm sequence. Thus *DBF3/PRP8* is a unique gene encoding a large protein that is highly conserved in a wide range of eukaryotes. From the study of the gene in yeast it appears to have a role in the cell cycle as well as the previously characterised role in pre-mRNA splicing.

MATERIALS AND METHODS

Yeast strains

L149-7B	*MATa dbf3-1 trp-289 ura3-52*
J219-3D	*MATa arg4 his7-2 leu2-3 spo12D::TRP1 trp1-289*
JL130	*MATa ade1 ade2 dna39-1 gal1 his7-2 lys2 tyr1 ura1*
JL2U	*MATa ade1 ade2 dna39-2 gal1 his7-2 lys2 tyr1 ura1*
JL342	*MATa ade1 ade2 dna39-3 gal1 his7-2 lys2 tyr1 ura1*
L149a	*MATa ade1 dbf3-1 his7-2 lys1 trp1-289 ura3-52*
JL1U	*MATa dna39-1 his7-2 trp1-289 ura3-52*
JL2U	*MATa ade1 dna39-2 his7-2 trp1-289 ura3-52*
JL3U	*MATa ade1 dna3-3 his7-2 leu2-3 tyr1 trp1-89*
CG378/CG379	*MATa/α ura3/ura3 leu2/leu2 trp1/trp1 ade5/+ +/his7*
D273	*MATα ade his1 trp2*
SPJ8-31	*MATα prp8-1 ura3 leu2 his3*
SPJ8-2	*MATa prp8-1 leu2 trp1 ura3*
J16a	*MATa prp8-1 ade1ade2 ura1 his7 lys2 tyr1 gal1*
JDY8-22	*MATα prp8-2 ura3 leu2 lys2 his3*
JDY8-31	*MATα prp8-3 ura3 his3*
DJY76	*MATα prp8-7 leu2 ura3 his7 tyr1*
JDY8-81	*MATα prp8-8 ura3 leu2 trp1 his3*

Strains JL130, JL253 and JL342 were obtained from Alan Wheals, University of Bath. CG378 and CG379 were from Dr C.Giroux, Detroit. The *prp8* strains were kindly provided by Andrew Brown and Jean Beggs, University of Edinburgh. Strains L149a, JL1U, JL2U, JL3U and J219-3D were constructed during this study. Construction of the *spo12Δ::TRP1* genotype has been described (27).

Media and general methods

YPD (28) and YNB (Difco) media were used as rich and minimal medium, respectively. Cell numbers were determined by use of a Coulter Counter (Coulter Electronics, Dunstable, UK). Plasmids were introduced into yeast treated with lithium acetate (29). Preparation of cells for flow cytometry has been described previously (27).

Cloning and disruption of the *DBF3/PRP8* gene

Seven plasmids that complemented the temperature sensitive growth of the *dbf3-1* strain L149-7B were isolated from a clone bank of yeast chromosomal DNA in vector YEp24. Six of these plasmids rescued the *dbf3-1* temperature-sensitivity efficiently and restriction analysis confirmed that they contained DNA from the same genomic region. The seventh plasmid rescued the *dbf3-1* defect weakly and was found to contain the *SSB1* gene (see Results). For deletion of the chromosomal *DBF3* gene, YEp24 containing a 9.8 kbp region of the *DBF3* locus was digested with *Bam*HI to remove some 6.0 kb of the *DBF3* ORF and then religated. The truncated *DBF3* gene was removed from the YEp24 vector by digestion with *Sal*I and *Cla*I and inserted between the *Sal*I and *Cla*I sites located in the polylinker of a modified pBluescriptKS vector (Stratagene) to form plasmid pKS-*dbf3Δ*. pKS-*dbf3Δ* was digested with *Bam*HI and the *URA3* gene was inserted to form plasmid pKS-*dbf3Δ::URA3*. This plasmid was then digested with *Eco*RI and the resulting fragment containing *URA3* flanked by *DBF3* sequences was used to transform the diploid strain CG378/CG379. Integration of the fragment and hence disruption of one chromosomal copy of the *DBF3* gene was confirmed by Southern hybridisation analysis.

α-Factor synchronisation

Cultures to be synchronised by α-factor were grown to mid-logarithmic phase in YPD (5×10^6 to 1×10^7 cells/ml) and α-factor added to a final concentration of 2.5 μg/ml. Incubation was continued until the percentage of budded cells had declined to below 5%. The α-factor was removed by filtration and washing. The cells were resuspended at an appropriate density in fresh medium, prewarmed to either 25 or 37°C as required.

Transposon sequencing and mutagenesis system

Generation of Tn1000 transposon insertions into target plasmids and use as a sequencing strategy was essentially as described (30). An extension of this method was used to locate the *dbf3-1* rescuing activity to the *SSB1* gene within the seventh plasmid (31).

RESULTS

Cloning and genetic mapping of the *DBF3/PRP8* gene

The wild type *DBF3* gene was cloned by complementation of the *dbf3-1* ts mutation and localised to a 9.4 kb region contained on a 9.8 kb insert (Fig. 1). Northern hybridisation analysis revealed a single transcript of 7.8 kb (results not shown) confirming the large size of the gene. The identity of the cloned DNA as the authentic *DBF3* locus was confirmed by integrative mapping (32). *DBF3* was located to chromosome *VIII* by hybridisation to a yeast pulsed-field chromosome blot (33). This was confirmed by genetic mapping, which showed that *DBF3* is linked to *SPO12* (16 parental ditypes, 0 non-parental ditypes and 4 tetratypes). Use of standard equations places *DBF3* 10 centimorgans from *SPO12* on the right arm of chromosome *VIII*. *DBF3* showed no linkage with *ARG4*, thus it is unclear on which side of the *SPO12* gene the *DBF3* gene is located. None of the mutations previously mapped in this region have phenotypes similar to *dbf3*. Thus, they are unlikely to be in the same gene. Determination of the nucleotide sequence (see below) showed that the *DBF3* reading frame starts 221 nucleotides downstream of *CDC23*, both genes being transcribed in the same direction.

The sequence of *DBF3* shows it to be identical to *PRP8*

Approximately 7845 bp of DNA containing the *dbf3-1* complementing activity was sequenced on both strands. One large open reading frame (ORF) of 7239 nucleotides was found (GenBank accession number L29421), capable of encoding a protein of 2413 amino acids with a calculated molecular weight of 268 kda (Fig. 2). The 3′ end of the *CDC23* gene (34) was found 221 nucleotides upstream of *DBF3/PRP8*. The predicted Dbf3/Prp8 protein is highly hydrophilic and has an estimated pI of 7.85. Interestingly, within the 78 residues of the N-terminal region there are four runs containing 5, 8, 7 and 7 proline residues. There is also a potential nuclear localisation signal (35) located downstream of the runs of proline residues at positions 109–124.

After we had completed sequencing the *DBF3* gene, the *PRP8* sequence (36) became available in the databases, and we found that *DBF3* and *PRP8* (*RNA8*) were one and the same gene. Although the two predicted proteins Dbf3 and Prp8 have identical numbers of amino acid residues, there are a number of minor differences. There is a frame shift that causes a difference between the two proteins at residues 386–419. This may be a genuine polymorphism since either reading of the amino acids in this region is equally homologous to the corresponding region of the worm gene (see below). Additionally, there are two further single amino acid substitutions at positions 1132 and 1566 of the protein.

The *DBF3/PRP8* gene is essential for mitotic growth

Isolation of a ts mutation does not necessarily prove that the gene is essential for growth. We therefore disrupted one chromosomal copy of the *DBF3* gene in a diploid strain using *URA3* (Fig. 1B) and following sporulation asci were dissected. Of ten tetrads, eight contained only two viable spores and two contained only one viable spore. All viable spores were *Ura*$^-$. Some non-viable spores germinated to produce a single dumbbell-shaped cell, others formed a microcolony of four to several hundred cells after 7 days incubation. Microdissection of these cells showed that a small proportion were capable of further cell division but required up to 48 h to do so. The daughter cells produced from these divisions were very small and rarely divided themselves. Thus *DBF3* is functionally required for mitotic growth.

The *dbf3-1, dna39 and prp8* mutations are allelic

We were surprised by the discovery that *DBF3* and *PRP8* were the same gene since we had previously found that the *dbf3-1* allele complemented the *rna8-1/prp8-1* ts defect and that the *dbf3-1* mutant appeared to have no effect on RNA synthesis at 37°C (18,19). We therefore repeated the complementation tests by mating a *dbf3-1* strain with three different strains containing the *prp8-1* allele and four other *prp8* alleles. All three *dbf3-1/prp8-1* diploids were capable of growth at 37°C confirming our earlier result. However, crosses between *dbf3-1* and the four other *prp8* alleles resulted in diploids unable to grow at 37°C confirming their allelism (Table 1). One of the *prp8-1/dbf3-1* diploids that grew at 37°C was sporulated and tetrads were dissected. Amongst 39 spores only one was a recombinant capable of growth at 37°C, consistent with the two mutations being in the same gene. The

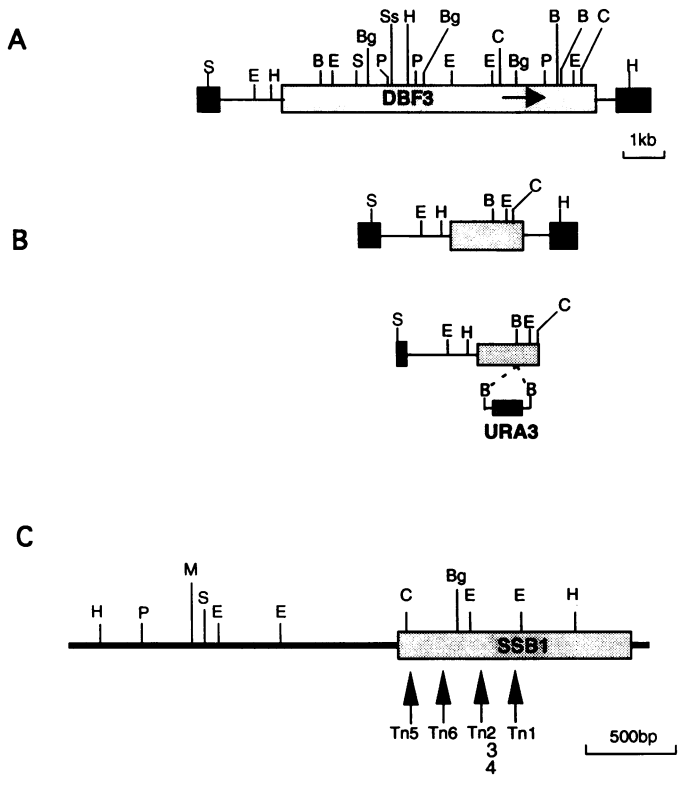


Figure 1. Restriction map of *DBF3* and *SSB1*, and the disruption strategy for *DBF3*. (**A**) Partial restriction map of *DBF3*. Abbreviations for restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Ss, *Sst*I. The direction of transcription is indicated by an arrow. (**B**) Disruption of *DBF3* by *URA3* as described in Materials and Methods. (**C**) Partial restriction map of the *SSB1* gene and flanking sequences. The insertion sites of the six Tn1000 transposons within the *SSB1* gene which destroyed suppressor activity are indicated by arrows.

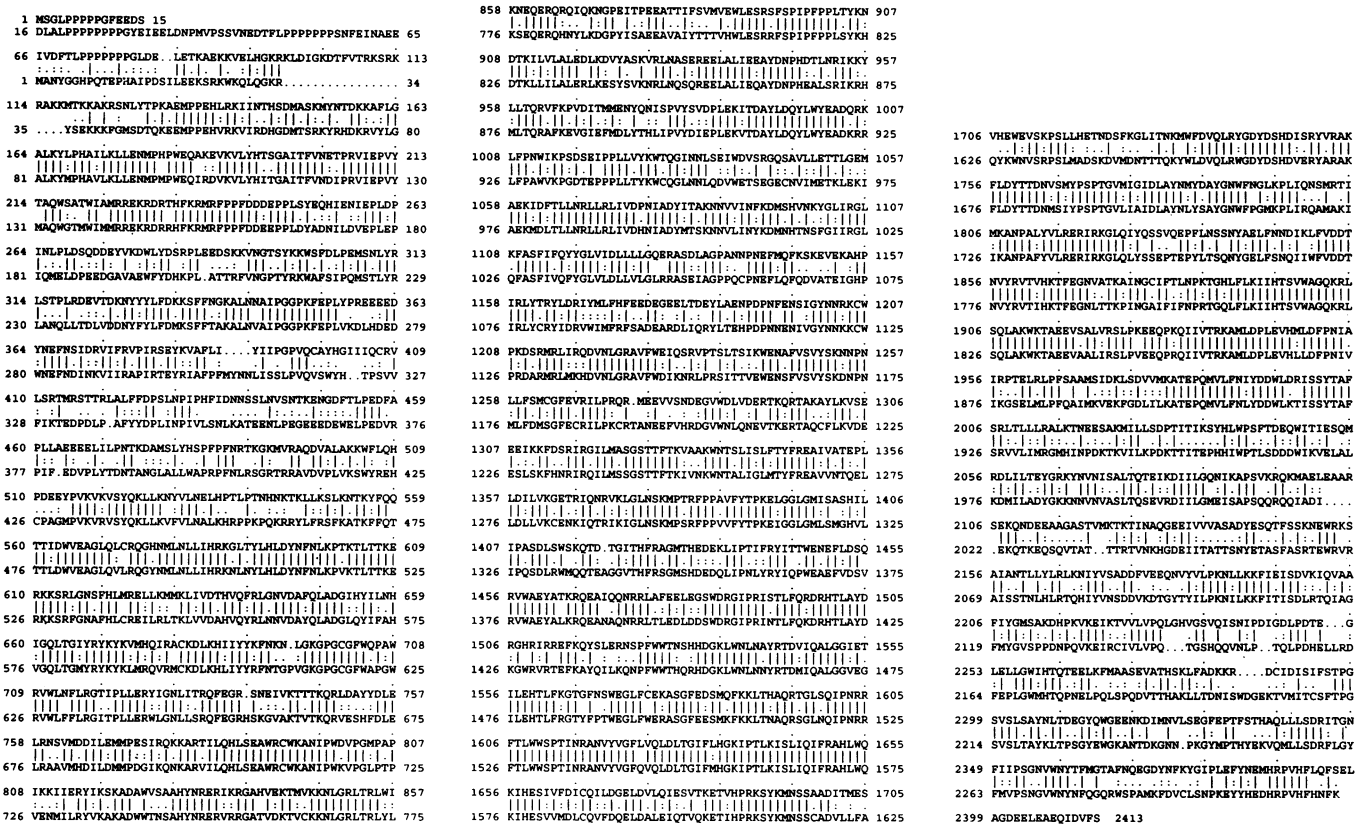


Figure 2. Alignment of the predicted yeast Dbf3 protein sequence with the homologous predicted protein from *C.elegans*. The upper line shows the sequence of the yeast protein and the lower line shows the sequence of the worm protein. Vertical lines indicate amino acid residues that are identical between the two sequences, and colons indicate conserved residues. The *DBF3* nucleotide sequence has GenBank accession number L29421.

ability of the *dbf3-1* allele to complement the ts defect of the *prp8-1* allele therefore indicates intra-allelic complementation and suggests that like calmodulin the Dbf3/Prp8 protein may contain independently functional domains (37).

The three *dna39* mutations were isolated in a separate screen for ts mutants defective in DNA synthesis (26). Transformation of *dbf3-1* and the *dna39* strains with the cloned *DBF3/PRP8* gene rescued the ts defect of all four mutants equally well and complementation tests between the strains confirmed their allelism.

DBF3/PRP8 is a highly conserved eukaryotic gene

Searches of the GenBank and EMBL databases with the predicted amino acid sequence of the Dbf3/Prp8 protein revealed significant homology to a predicted protein from *Caenorhabditis elegans* (worm) derived from DNA sequence contained between base pairs 6247 and 14 007 of cosmid C50C3, accession number L14433 (38). Eight predicted exons were found within the region 7074 to 14 007 corresponding to this putative protein labelled C50C3.6, which is of unknown function. In addition, on the basis of homology to Dbf3, there appears to be a ninth exon in the worm sequence between positions 6190 and 7002. The nine exons together predict a protein with about 58% sequence identity to Dbf3 over 2300 amino acids (Fig. 2). The homology extends from position 83 in the Dbf3/Prp8 sequence to the end of both genes, but does not include the proline rich domains found in the N-terminal region of Dbf3.

Similarly, the expressed sequence tag (EST) database (National Center for Biotechnology Information), which contains mostly human transcribed sequence fragments of unknown function, was searched and two human protein sequences with significant homology to Dbf3 were found (39). These two EST sequences correspond to residues 1445−1556 and 1989−2106 of the Dbf3/Prp8 protein, and it is therefore probable that they are different parts of the same human gene. The alignment of all three genes, human, worm and yeast (Fig. 3), showed that the first 19 amino acid residues of the partial human sequence (Fig. 3A) were not homologous to either worm or yeast, and therefore it is not certain that they belong here. Thus, ignoring these 19 amino acids, the sequences each contain 211 amino acids that can be compared between the three organisms; yeast and worm are 58% identical, yeast and human are 61% identical, whilst human and worm are 87% identical over these regions. Since the identity between the yeast and worm sequences in these regions is representative of the overall identity between the yeast and worm sequences, it is probable that 58 and 87% are good estimates of the overall level of identity with the human Dbf3/Prp8 protein.

The *dbf3-1* mutation can be suppressed by high copy expression of *SSB1*

As described in Materials and Methods, one of the seven library plasmids obtained during the search for the *DBF3* gene only weakly complemented the *dbf3-1* defect at 37°C and contained

Table 1. Complementation between *dbf3-1* and alleles of *prp8*

Strain	Genotype	Growth of Diploid at 37°C	Genotype	Strain
D273	*dbf3-1*	+	*prp8-1*	SPJ8-31
D273	*dbf3-1*	+	*prp8-1*	SPJ8-2
D273	*dbf3-1*	+	*prp8-1*	J16a
L149a	*dbf3-1*	-	*prp8-2*	JDY8-22
L149a	*dbf3-1*	-	*prp8-3*	JDY8-31
L149a	*dbf3-1*	-	*prp8-7*	DJY76
L149a	*dbf3-1*	-	*prp8-8*	JDY8-81
L149-7B	*dbf3-1*	-	*dna39-1*	JL130
L149-7B	*dbf3-1*	-	*dna39-2*	JL253
L149-7B	*dbf3-1*	-	*dna39-3*	JL342

The *dbf3-1* mutation in the genetic backgrounds shown on the left hand column was crossed with the *prp8* and *dna39* alleles shown on the right. Growth of the resulting diploids at 37°C is shown in the centre column.

a different genomic insert to the plasmids containing the authentic *DBF3/PRP8* gene. Using combined transposon mutagenesis and sequencing (31) the suppressor activity of this plasmid was mapped to a region of the insert that contained the *SSB1* gene (Fig. 1), a member of the hsp70 heat shock protein family (40). Suppression of *dbf3* by *SSB1* under these conditions is specific for the *dbf3-1* allele. A number of *prp8* and *dna39* alleles were tested and none were rescued by *SSB1* even at a temperature of 35°C. This may suggest a specific interaction between Ssb1 and the defective protein encoded by the *dbf3-1* allele, but it remains uncertain whether or not they normally interact during cell division in wild-type yeast. A link between heat shock proteins and pre-mRNA splicing has previously been described (41,42), however this is the first instance of suppression of a ts defect of a snRNP protein by over-expression of a heat shock protein.

The *dbf3-1* mutation confers a cell cycle arrest phenotype

Our previous description of the *dbf3-1* mutant focused largely on its effect on DNA synthesis (18,19). Since a connection between pre-mRNA splicing and the cell cycle, suggested by the allellism of *PRP8* and *DBF3*, was entirely unexpected, we have further characterised the *dbf3-1* phenotype to establish clearly that it has a role in the cell cycle. This was also important as none of the *prp8* alleles that we examined showed a uniform cellular morphology at 37°C like that of *dbf3* (a 'dumbbell', with an enlarged bud attached to the mother cell, see Fig. 4D).

To examine the cell cycle arrest in *dbf3-1*, log phase cultures of *dbf3-1*, *prp8-1* and a wild type strain were grown at the permissive temperature then shifted to 37°C for 4 h. Samples were taken at hourly intervals to determine cell density, proportion of budded cells and the proportion of cells with divided chromatin (Fig. 4).

In the *dbf3-1* culture, 46% of the cells completed cell division after shifting to the restrictive temperature, as expected for a cell cycle mutant (data not shown). After 4 h at 37°C, 75% of cells had arrested with buds (Fig. 4C) and some 75% of cells contained a 2C content of DNA (Fig. 4C and A). However, essentially none of the cells had divided chromatin (Fig. 4B and D). They had therefore not proceeded to nuclear division, in spite of the

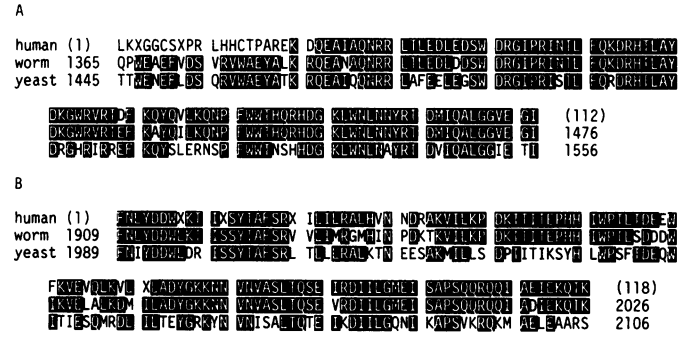
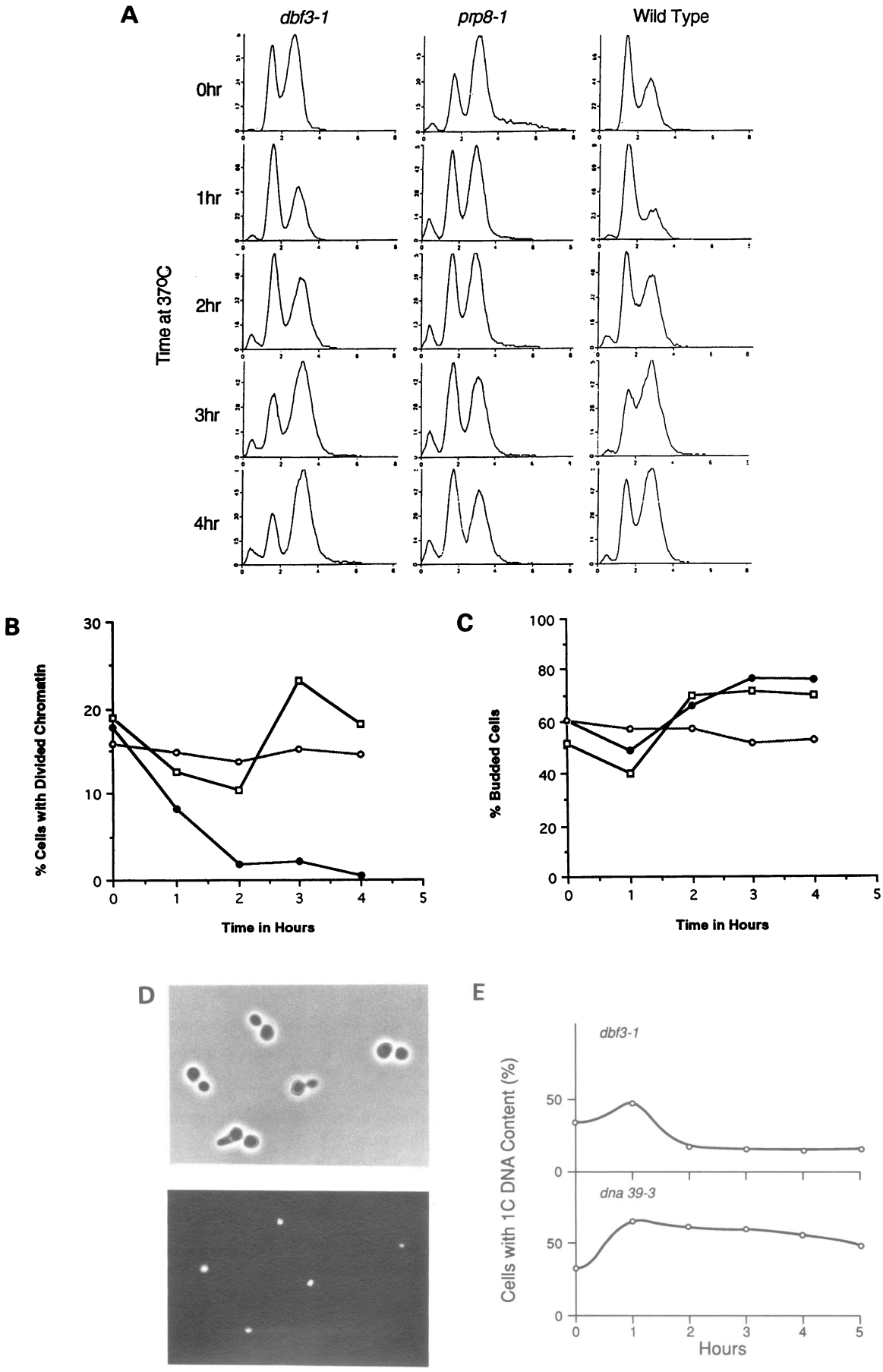


Figure 3. Alignment of partial Dbf3 sequences from human, worm and yeast. Protein sequences deduced from human nucleotide sequences (**A**) EST05386 and (**B**) EST05073 were aligned with the corresponding regions of the yeast and worm protein sequences.

apparent 2C DNA content, and were arrested at a cell cycle stage before the metaphase to anaphase transition.

In the *prp8-1* culture, cell division ceased rapidly and there was no evidence for cell cycle stage specific arrest of divsion. This resulted in arrested cells in which the nuclear morphology and DNA content remained in its original state at the time of the temperature shift. Thus *prp8-1* results in a rapid cessation of the cell cycle at 37°C, independent of the cell cycle stage in progress.

In contrast to the *dbf3-1* culture, cell numbers in the *prp8-1* culture increased by only 11% after shifting to 37°C. This raised the possibility that part of the 46% increase in cell numbers in the *dbf3-1* culture at 37°C could have resulted from leakiness of the *dbf3-1* allele. We therefore extended the FACS analysis to *dna39-3*, an allele that we had found to be a very tight temperature sensitive mutant (Fig. 4E). This resulted in a higher proportion of the population accumulating with 1C DNA than occurred with *dbf3-1*, consistent with a function early in the cell cycle (see below). Despite this apparent cell cycle specific block


A
dbf3-1
prp8-1
Wild Type
0hr
1hr
2hr
3hr
4hr
Time at 37°C
B
% Cells with Divided Chromatin
Time in Hours
C
% Budded Cells
Time in Hours
D
E
dbf3-1
dna 39-3
Cells with 1C DNA Content (%)
Hours

in *dna39-3*, the cells did not assume the expected terminal phenotype of 'dumb-bells' but were of mixed morphology even after a protracted incubation at 37°C.

Wild-type cells continued dividing after the temperature shift but a transient delay in budding occurs presumably as a result of a heat shock effect which is known to specifically delay the cell cycle at START (43). This transient effect was also observed in the *dbf3-1* culture.

Thus, the *dbf3-1* allele clearly has a cell cycle arrest phenotype but the phenotype of *prp8-1* is quite different.

The *dbf3* mutant has a specific defect early in the cell cycle

With ts mutants having a cell cycle defect, it is possible to determine the time in the cell cycle at which the gene product functions. This time, known as the 'execution point' is characteristic for particular cell cycle mutants and confirms a cell cycle role for the gene product in question. In practice one normally determines the final time in the cell cycle when the particular protein carries out an essential function. To do this synchronous cultures of *dbf3-1* and *dna39-3* mutants were

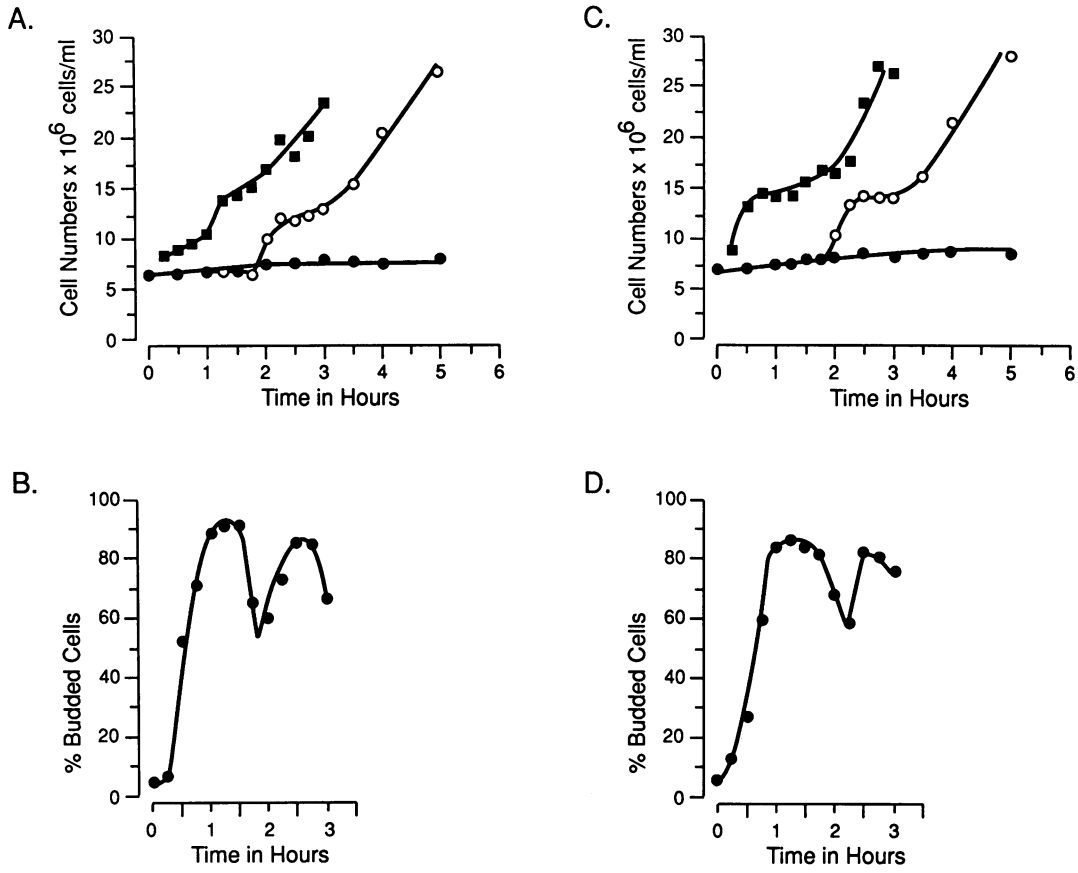


Figure 5. Execution point analysis of the *dbf3-1* and *dna39-3* Alleles. Mid-log phase cultures of the *dbf3-1* (strain L149a, panels **A** and **B**) and *dna39-3* (strain JL342, panels **C** and **D**) were grown at 25°C in YPD and synchronised using α-factor. The progress of the synchronous cell cycles following removal of α-factor were monitored by determining the percentage of budded cells at 25°C (panels B and D). After release from α-factor, cell numbers were measured (panels A and C) during further incubation at 25°C (open circles) or 37°C (closed circles). Additionally, further samples were transfered at 15 min intervals from the 25°C culture to 37°C and cell numbers were determined after a further 4 h incubation (squares) to determine the execution point.

Figure 4. DNA synthesis in asynchronous *dbf3-1*, *prp8-1* and wild type cultures at the permissive and restrictive temperatures. The *dbf3-1* strain (L149-7B), *prp8-1* strain (SPJ8-31) and wildtype strain (CG378) were grown at 25°C for approximately 10 generations to mid-log phase in minimal medium. The cultures were then shifted to 37°C and samples taken at hourly intervals for determination of DNA content per cell by flow cytometry (panel **A**). The percentage of cells with divided chromatin in each strain was determined microscopically using propidium iodide stained cells and is shown in panel **B** (filled circles, *dbf3-1* culture; open circles, *prp8-1* culture; squares, wild type culture). The percentage of budded cells in each strain was also calculated and is shown in panel **C** (figure legend as in panel B). *dbf3-1* dumbbell-shaped cells formed after 4 h holding at 37°C contain a single undivided nucleus (panel **D**). The cells were stained with propidium iodide as for flow cytometry and then photographed under light and UV illumination. Panel **E** shows the proportion of 1C cells that accumulate at 37°C in a culture of *dbf3-1* compared to *dna39-3*. The 1C cells were calculated from a FACS analysis like that in panel A. Note the *dbf3-1* data is not derived from panel A but is an independent experiment.

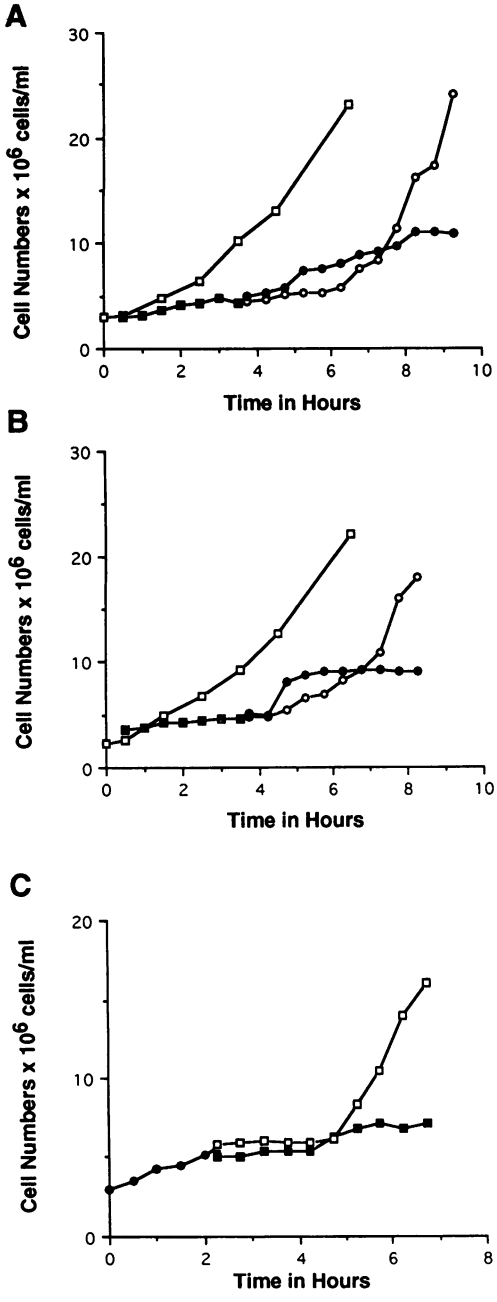


Figure 6. Reciprocal shift experiments between hydroxyurea and 37°C for the *dbf3-1 and dna39-3* Alleles. (**A**) A mid-log phase *dbf3-1* culture (strain L149-7B) grown in YPD at 25°C was divided into two; hydroxyurea was added to one of the cultures at a concentration of 300 mM (filled squares), and the other culture was incubated in the absence of hydroxyurea (open squares). After a further 3 h incubation the hydroxyurea was removed by filtration, the cells were washed with saline and the culture was split in two. Half of the cells were resuspended in prewarmed YPD at 37°C (filled circles), and the other half were resuspended in YPD at 25°C (open circles). The cultures continued incubation for a further 5.5 h and the cell numbers were determined at 30 min intervals. (**B**) The same experiment was carried out with a *dna39-3* culture (strain JL342), the only difference being that cell numbers were monitored for 4.5 h after removal of hydroxyurea. (**C**) As the second stage of the experiment in part (A), a mid-log phase *dbf3-1* culture (strain L149-7B) grown in YPD at 25°C was transferred to 37°C for one generation time (filled circles). The culture was then divided into two; hydroxyurea was added to one portion to 300 mM (filled squares), whilst the other portion received no hydroxyurea (open squares). Both cultures were transferred to 25°C and the cell numbers determined.

prepared at the permissive temperature using the α-factor method. After synchronisation samples were transferred to 37°C at regular timepoints during the ensuing cell cycle and the cell numbers measured to determine the proportion of cells that could complete the division cycle at the restrictive temperature. Cells that have passed the execution point prior to transfer to 37°C are able to divide, cells that have not passed the execution point do not divide. The percentage of budded cells in the main cultures at 25°C was also determined, both as a measure of synchrony and to indicate when DNA synthesis commenced as bud emergence is roughly coincident with initiation of S phase (44). Thus the time when the cells acquired the ability to divide at 37°C could be compared with the timing of S phase by a comparison with bud emergence.

Importantly, the samples from both cultures showed a clear, step-wise doubling of cell numbers when transferred to 37°C, indicative of a specific cell cycle defect (Fig. 5). With *dna39-3*, after only 45 min incubation at 25°C post α-factor release, cells had acquired the ability to divide at 37°C (Fig. 5C). At this time bud emergence in the culture was already 60% (Fig. 5D). This was consistent with a very early execution point at about the time of initiation of S phase. In contrast, *dbf3-1* cells were only able to divide at 37°C after 1 h 15 min prior incubation at 25°C, at which time budding was at a plateau of some 90% (Fig. 5A and B). This indicated an execution point close to the end of S phase. Thus, both of these execution point estimates support a role for the Dbf3 protein early in the cell cycle.

A simple explanation for the difference between the two alleles is that the mutant proteins have differing stabilities at 37°C. Alternatively, Dbf3 protein could have two functions, one of which is required at the beginning and one near the end of S phase, and that the different alleles affect one or other function more severely. To help determine the relationship between *DBF3* function and DNA synthesis, reciprocal shift experiments were performed between hydroxyurea (HU), an inhibitor of DNA synthesis, and the restrictive temperature. Inititally *dbf3-1* and *dna39-3* cultures were blocked with HU for one generation time at 25°C and then immediately transferred to 37°C on removal of HU. If the hydroxyurea-sensitive step (DNA synthesis) precedes Dbf3 function then the cells should be unable to divide, if it is after Dbf3 function the cells should undergo one cell division before arresting at 37°C. In this experiment, both strains showed a doubling in cell numbers after transfer to 37°C. In the case of *dna39-3* it was an almost exact doubling (Fig. 6B), whereas with *dbf3-1* the cell numbers more than doubled suggesting that this allele is somewhat leaky (Fig. 6A). Thus, the function of Dbf3 precedes the occurrence of complete DNA replication.

In the second part of the experiment a *dbf3-1* culture was held at 37°C for 2.5 h (over one generation time), before addition of HU and transfer to 25°C. The majority of cells (60%) did not divide in HU (even though 100% could recover in the absence of HU), indicating that in these cells the HU-sensitive step is dependent on prior Dbf3 function (Fig. 6C), consistent with the first part of the experiment. Presumably at 37°C some DNA must remain unreplicated even in cells with an apparent 2C DNA content as judged by the FACS analysis (Fig. 4A). However, 40% of the cells did divide in HU. Possibly the *dbf3-1* allele is leaky and allows a proportion of cells to fully replicate their DNA at 37°C. These cells would then be resistant to HU and would divide at 25°C resulting in the 40% increase in cell numbers. This result could not be confirmed with the *dna39-3*

strain, since incubation of this mutant at 37°C is rapidly lethal and very few cells divide on transfer to 25°C even in the absence of HU. This suggests that the defect in *dna39-3* mutant is irreversible.

DISCUSSION

We have found that the cell cycle mutation *dbf3-1* is an allele of *PRP8*, a U5 snRNP. This result was quite unexpected and led us to analyse the *dbf3-1* phenotype further to confirm that it does indeed display a specific cell cycle defect. The *dbf3-1* mutant was previously shown to arrest at 37°C with a cellular morphology typical of a cell cycle defect (18,19). Here we show that at the restrictive temperature at least 75% of cells accumulate with an approximately 2C content of DNA and all cells arrest with a single undivided nucleus. This is an important result showing that none of the cells progress into mitosis, consistent with a specific block in the cell cycle. The remaining cells arrested with a 1C content of DNA probably at START. Presumably, in these cells the defect in the Dbf3/Prp8 protein prevents entry into the cell cycle and may reflect differing levels of functional Dbf3/Prp8 protein amongst the mutant cells.

A specific cell cycle defect in *dbf3-1* cells is also indicated by the execution point analysis. At 37°C both *dbf3-1* and *dna39-3* have clear execution points so that their defective gene products are carrying out their essential functions at particular defined points in the cell cycle. Both execution points are early in the cell cycle, around about the time of S phase, but, unexpectedly, the *dna39-3* execution occurred earlier than that of *dbf3*. Consistent with an earlier execution point for *dna39-3*, a higher proportion of cells accumulate wih a 1C DNA content at 37°C than do with *dbf3-1*. This might reflect a difference in leakiness between the two alleles or, conceivably, the Dbf3/Prp8 protein might function at two different times in the cell cycle.

The *dbf3* mutant was originally identified amongst a group of mutants with S phase defects (18,19) and the *dna39* alleles were also identified in a screen for mutants defective in DNA replication (26). Our data with the DNA synthesis inhibitor hydroxyurea is also consistent with some role for Dbf3/Prp8 in or near S phase. In spite of some 75% of cells arresting with an apparent 2C DNA content, 60% of the cells remain sensitive to HU. Thus, some DNA in cells with an apparent 2C DNA content must remain unreplicated. This is consistent with the known behaviour of certain mutants defective in initiation of S phase (45). The other 40% of cells which completed the cell cycle in the presence of HU might indicate a degree of leakiness in *dbf3-1*, which allows some cells to complete DNA synthesis at 37°C.

In summary, *dbf3* clearly has a cell cycle phenotype with the defect occurring early in the cell cycle. The screens used to identify *dbf3* and *dna39*, together with the HU reciprocal shift experiments, suggest a role in S phase, possibly in initiation of DNA synthesis.

The sequence of the *DBF3/PRP8* gene shows four tracts of proline residues within 78 residues of the N-terminus. Polyproline sequences have been found in a number of transcriptional activators (46), suggesting that Dbf3/Prp8 could affect the transcription of genes required for S phase and that are expressed under cell cycle control in late G1, such as the S phase specific cyclins *CLB5* and *CLB6* (47–50). Importantly, these proline residues are not present in the worm sequence which may indicate that they are unnecessary for the pre-mRNA splicing function of the protein. Thus the polyproline sequences may be yeast specific and involved in the S phase function of the Dbf3/Prp8 protein. Certainly, the yeast U5 snRNP is larger than its mammalian homologue and thus may have additional functions (1).

Apart from the proline sequences, *Prp8/Dbf3* is a highly conserved eukaryotic protein. It has 58% identity with a *C.elegans* putative protein, spread over the entire 2300 amino acids of the protein, and 61% identity with the partial deduced sequence of a human protein. Furthermore, the *C.elegans* and human proteins are 87% identical. This level of homology is comparable to the cyclin dependent kinase $p34^{CDC28}$ which has 60.5% identity between yeast and human, and this includes the catalytic domain common to nearly all protein kinases. This similarity in protein sequence confirms the previous immunological data which showed that a Prp8 homologue is present in higher eukaryotes.

The discovery that *DBF3* and *PRP8* are the same gene raises the possibility that either pre-mRNA splicing plays a role in cell cycle progression, or that one or more of the snRNA complexes may have dual roles in cell cycle progression as well as splicing.

A role for pre-mRNA splicing in cell cycle progression would imply the existence of an intron-containing gene encoding a rate-limiting cell cycle protein. To explain the first cycle arrest at 37°C of *dbf3* mutants this protein would have to be unstable and synthesised *de novo* early in the cell cycle between START and the end of S phase. Only a limited number of known cell cycle proteins could fulfil these requirements, but none of them have been shown to contain an intron. However, studies using cycloheximide, a protein synthesis inhibitor, have shown that cell cycle progression requires protein synthesis in G1 (51) and also in two distinct intervals after G1 (52). If one of these unknown proteins is encoded by a gene containing introns then this could account for the cell cycle block in *dbf3*. A further prediction of this hypothesis is that other splicing and cell cycle mutants might be allelic. So far, this does not appear to be the case, with the possible exception described below.

The alternative hypothesis mentioned above is that certain snRNA complexes have a role in cell cycle progression that is independent of splicing itself. Unlike the *prp8* (*rna8*) mutation, which inhibits RNA synthesis (2,6,7), the *dbf3-1* allele has no detectable effect on gross RNA synthesis (18,19) and nonetheless results in a cell cycle stage-specific arrest at the restrictive temperature. Therefore the cell cycle phenotype of *dbf-1* cannot easily be explained as the secondary result of a primary defect in RNA synthesis or processing. One other *dbf* mutant, *dbf5*, is also allelic to a *prp* mutant, namely *prp3* (*rna3*) (18). Interestingly, this is a U4/U5 snRNP (2) and *DBF3/PRP8* is necessary for the assembly of the U4/U6.U5 triple snRNP complex on the pathway to splicosome assembly (9). The coincidence of cell cycle phenotypes caused by mutations in the genes encoding the snRNP's *DBF5/PRP3* and *DBF3/PRP8* points to the U4/U6.U5 triple snRNP as the complex with a potential role in the regulation of S phase. Conceivably, the formation of the complex is necessary early in each cell cycle and this is co-ordinated with S phase by the dual function of Dbf3/Prp8 and Dbf5/Prp3. Dbf3/Prp8 is certainly a very large protein with ample structural potential to be multifunctional. Consistent with this the *dbf3-1* allele complements the ts defect of the *prp8-1* allele suggesting that their defects lie in different functional domains.

Lastly, it is worth drawing attention to the apparent link between carbon catabolite metabolism and pre-mRNA splicing

(see Introduction). Taken together with the connection between splicing and the cell cycle, this might suggest that RNA metabolism has to be co-ordinated with other events within the cell. If so, further links between splicing and other cell biological pathways might be found.

ACKNOWLEDGEMENTS

We thank Dr R.Singer, Halifax, for informing us that *dbf3* and *dna39* mutations are allelic, Chris Atkins, NIMR, for his help with the flow cytometry, and Jean Beggs and Andrew Brown for *prp8* strains.

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