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Autoregulation of expression of the yeast Dbp2p 'DEAD-box' protein is mediated by sequences in the conserved *DBP2* intron

I.Barta and R.Iggo[1]

Swiss Institute for Experimental Cancer Research (ISREC), 1066 Epalinges, Switzerland

[1]Corresponding author

The human p68, *Saccharomyces cerevisiae DBP2* and *Schizosaccharomyces pombe dbp2* genes are closely related members of the 'DEAD-box' RNA helicase superfamily. All three genes contain an intron at a conserved site in RNA helicase motif V. The *S.cerevisiae* intron is unusual both for its position near the 3′-end of the open reading frame and for its size, 1001 nucleotides. We show here that precise deletion of the intron has no effect on cell viability but leads to an increase in Dbp2p protein expression. Inefficient splicing due to the size of the intron can not account for this difference because the intron is efficiently spliced in Dbp2p-deficient cells. Instead, there is a reciprocal relationship between the amount of Dbp2p in the cell and the efficiency with which *DBP2* intron-containing genes are expressed. Inactive Dbp2p mutants are efficiently expressed from *DBP2* intron-containing plasmids, and fragments of the *DBP2* intron confer Dbp2p-responsiveness on heterologous reporter introns. This suggests that there is an intron-mediated negative feedback loop regulating *DBP2* expression, and provides a possible explanation for the retention of such an unusual intron in *S.cerevisiae.*

Keywords: *DBP2*/DEAD-box protein/p68/RNA helicase/ RNA processing

Introduction

The putative RNA helicases of the 'DEAD/H' family are ubiquitous proteins which participate in diverse processes including RNA splicing, ribosome assembly, initiation of translation, spermatogenesis and embryogenesis (reviewed by Linder *et al.*, 1989; Wassarman and Steitz, 1991; Schmid and Linder, 1992). One of the best characterized members of the family is the human nuclear antigen p68, which possesses both RNA-dependent ATPase (Iggo and Lane, 1989) and ATP-dependent RNA helicase activity (Hirling *et al.*, 1989). Despite being well studied biochemically, the biological function of p68 is unknown, and to help address this problem we previously cloned p68-like genes from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Iggo *et al.*, 1991).

The yeast and human genes contain an intron whose position in RNA helicase motif V has been precisely conserved (Gorbalenya *et al.*, 1989; Iggo *et al.*, 1991), and the intron in the *S.cerevisiae* gene, *DBP2,* is remarkable not only for its position near the 3′-end of the gene but also for its length, 1001 nucleotides, which is twice that of any other known intron in *S.cerevisiae* (Rymond and Rosbash, 1992). These unusual features suggest that the intron has a function: it could encode a separate gene or participate in regulation of *DBP2* expression, or both. The intron does not contain conserved sequences or long open reading frames suggesting the presence of intron-encoded proteins. However, many snoRNAs have been identified in the introns of nuclear genes, particularly nucleolar protein genes (reviewed by Sollner-Webb, 1993), and mammalian p68 is present in pre-nucleolar bodies (Iggo *et al.*, 1991). Although sequence analysis identifies no known snoRNA homologues in the *DBP2* intron, there are snoRNA C and D boxes (potential fibrillarin binding sites, Baserga *et al.*, 1991) at positions 805 and 846 in the intron.

Alternatively, the *DBP2* intron could have a regulatory role. This is attractive given the potential for an RNA helicase to modify the secondary structure of its own pre-mRNA. Secondary structure in pre-mRNA can promote splicing by effectively reducing the distance between a branch point and 3′-splice site (Chebli *et al.*, 1989; Deshler and Rossi, 1991) and it can inhibit splicing by sequestering splicing signals (Eperon *et al.*, 1988; Goguel *et al.*, 1993). Five putative RNA helicases are known to be required for RNA splicing [*PRP2* (Chen and Lin, 1990), *PRP5* (Dalbadie and Abelson, 1990), *PRP16* (Burgess *et al.*, 1990), *PRP22* (Company *et al.*, 1991) and *PRP28* (Strauss and Guthrie, 1991), reviewed by Wassarman and Steitz, 1991] and in several cases they are thought to have a regulatory role (Burgess and Guthrie, 1993).

Autoregulation of splicing has been documented for the ribosomal protein Rpl32p, which blocks splicing of its own pre-mRNA by binding to a hairpin structure encompassing the 5′ splice site (Dabeva *et al.*, 1986; Eng and Warner, 1991; Vilardell and Warner, 1994). Autoregulation of splicing has also been shown for the *Drosophila suppressor of white apricot*, $su(w^a)$, and *transformer-2, tra-2,* genes (reviewed by Balvay *et al.*, 1993). Intriguingly, the introns of *Xenopus* L1 ribosomal protein have a dual function: they encode U16 and U18 snoRNAs, and participate in intron-mediated autoregulation of L1 expression (Caffarelli *et al.*, 1987; Fragapane *et al.*, 1993; Prislei *et al.*, 1993).

The *DBP2* intron could also play a regulatory role by affecting *DBP2* pre-mRNA stability. Osteoblast lineage-specific expression of the liver/bone/kidney alkaline phosphatase gene may result from cell type-specific intron-mediated RNA instability (Kiledjian and Kadesch, 1991), and several RNA helicases, including human p68, have recently been shown to influence RNA stability in *Escherichia coli* (Iost and Dreyfus, 1994).

Here, we report that deletion of the *DBP2* intron results in an increase in the level of Dbp2p mRNA and protein.

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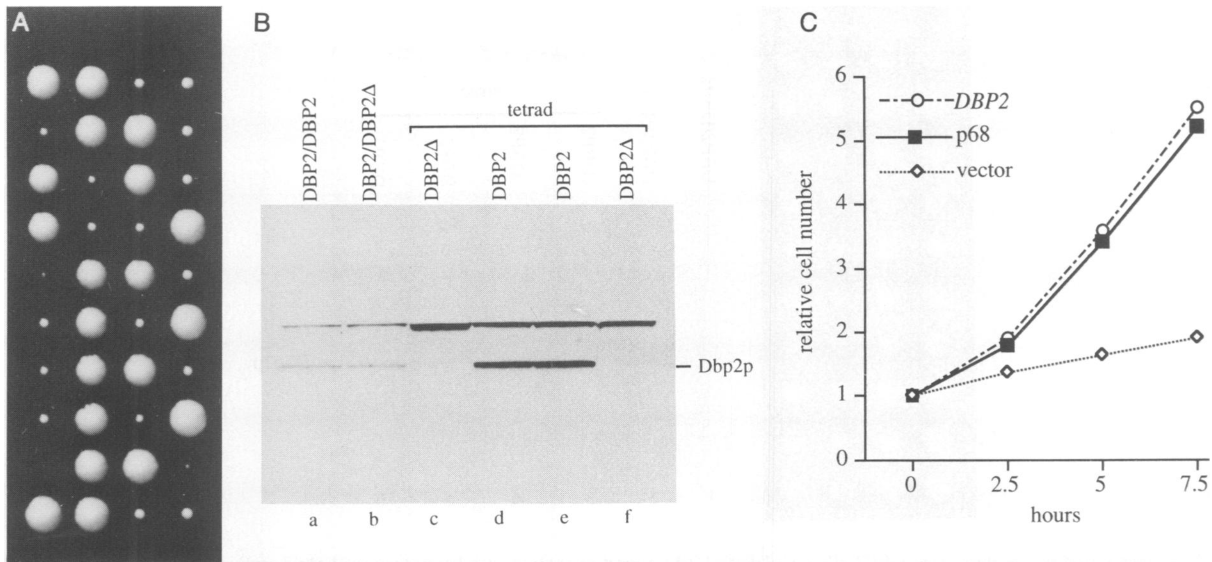


Fig. 1. Characterization of the *DBP2* null allele (*'DBP2Δ'*). (**A**) Tetrad dissection of a heterozygous diploid strain (*DBP2/DBP2Δ*, yIB12) after growth at 35°C for 4 days. (**B**) Western blot probed with MaD1 (a, parental diploid; b, heterozygous diploid; c–f, haploid progeny of a heterozygous tetrad). (**C**) Complementation of the *DBP2* null by *DBP2* and human p68 (pIG79 and pIB13 in yIB12/1). *DBP2* and p68 were expressed from the *GAL1* promoter at 32°C in a haploid *DBP2* null strain transferred to medium containing galactose at time 0. The control strain (◇) contains parental vector (P2 in yIB12/1).

This is consistent with the intron having a role in regulating *DBP2* expression, and we show using heterologous reporters that a 552 nucleotide fragment of the *DBP2* intron confers Dbp2p-responsiveness on an artificial intron.

Results

Deletion of the DBP2 gene renders yeast cold-sensitive for growth

A diploid strain with one copy of the *DBP2* gene deleted was constructed by one-step gene replacement (Rothstein, 1991) and examined by tetrad dissection (Figure 1A and B). *DBP2Δ* spores form microcolonies with a cold-sensitive growth defect: the doubling time in rich medium is 5 h at 35°C, 12.5 h at 25°C and 41 h at 19°C, compared with 1.7, 2.2 and 3.9 h, respectively, for wild-type cells from the same cross. Dbp2p was detected in Figure 1B with MaD1, an antibody recognizing the 'DEAD' motif (Iggo *et al.*, 1990); the upper band is probably Ded1p (Jamieson *et al.*, 1991), which is also recognized by the antibody. The growth defect is rescued by plasmids expressing *S.cerevisiae DBP2*, *S.pombe dbp2* and human p68 (Figure 1C and data not shown).

DBP2 expression is regulated by sequences within its intron

The 1 kb intron in RNA helicase motif V of the *DBP2* gene was precisely deleted by two-step gene replacement (Rothstein, 1991). Cells containing the *DBP2* cDNA allele are viable and grow normally at all temperatures tested (Figure 2A, and data not shown). Western blotting shows that the cDNA allele gives a higher level of Dbp2p protein expression than the intron-containing wild-type allele (Figure 2B; the upper, cross-reacting band acts as a loading control). The increase in protein is paralleled by a 4-fold increase in the *DBP2* RNA level (Figure 3), suggesting that the difference in protein level is not due to more efficient translation.

Dbp2p is more efficiently expressed from plasmids containing the intronless DBP2 gene

The lower level of Dbp2p expression from the intron-containing *DBP2* allele is consistent with either inefficient splicing due to the size of the intron or regulation involving sequences within the intron. To distinguish between these possibilities, feedback of Dbp2p on its own expression was examined using Dbp2p tagged at the C-terminus with the KT3 epitope (PPEPET, MacArthur and Walter, 1984). The modified protein is still active since it can rescue the cold-sensitive growth defect of the *DBP2Δ* strain (data not shown). As with the endogenous gene (Figure 2B), the intron prevents high level expression of Dbp2p from a plasmid (Figure 4, compare lanes a and b).

Mutant DBP2 alleles containing the intron are efficiently expressed

If the poor expression of intron-containing *DBP2* alleles is caused by inefficient splicing, the intron should reduce expression of inactive *DBP2* mutants in the same way that it reduces expression of wild-type *DBP2*. In contrast, if the intron participates in a feedback loop, it should not affect expression of inactive mutants. Two mutants were tested, a lysine to asparagine mutation in the ATP binding site (GKT to GNT) and a C-terminal deletion which removes the glycine/arginine-rich domain (RGG^-). The GNT mutant is unable to complement the cold-sensitive growth defect of the *DBP2Δ* strain in all conditions tested, whereas the RGG^- mutant is partially active since it complements the deletion when expressed at high but not low level (data not shown). Figure 4 (lanes c–f) shows that the intron has no effect on the level of expression of either mutant. Thus the intron does not affect expression

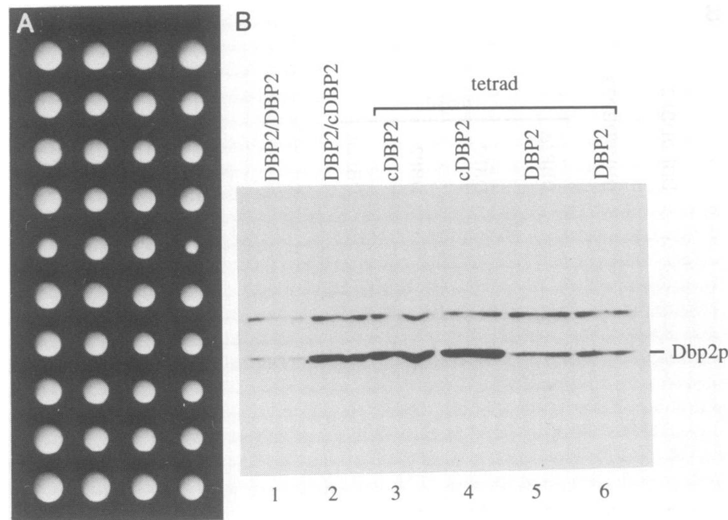


Fig. 2. Characterization of the intronless *DBP2* allele (*'cDBP2'*). (**A**) Tetrad dissection of a heterozygous diploid strain (*DBP2/cDBP2*, yIB15) after growth at 32°C for 3 days. (**B**) Western blot probed with MaD1 (1, parental diploid; 2, heterozygous diploid, 3–6, haploid progeny of a heterozygous tetrad).

purely by causing inefficient splicing. Instead, it mediates some form of autoregulation of Dbp2p expression.

Exogenous Dbp2p suppresses endogenous Dbp2p expression

If the intron autoregulates *DBP2* expression, the level of endogenous Dbp2p should fall in cells overexpressing *DBP2* from a plasmid. A wild-type strain was therefore transformed with vectors constitutively expressing wild-type or mutant *DBP2* cDNA, and the Dbp2p level was examined by Western blotting (Figure 5). Exogenous Dbp2p can be distinguished from endogenous protein because of its lower mobility due to the presence of the KT3 epitope tag (note that the MaD1 antibody was used for the blot). Figure 5 shows that high level expression of exogenous Dbp2p abolishes endogenous Dbp2p expression (compare lanes a and b), but the GNT and RGG⁻ mutants do not (lanes c and d), consistent with the feedback model.

Endogenous Dbp2p suppresses exogenous Dbp2p expression

Next, we tested whether endogenous Dbp2p can dictate the level to which exogenous Dbp2p can be expressed from a plasmid containing the KT3-tagged, intron-containing *DBP2* allele. Strains containing the deletion, wild-type and cDNA alleles at the chromosomal *DBP2* locus were used, since these express no Dbp2p, normal and elevated levels of Dbp2p, respectively. In the null strain the exogenous protein is strongly expressed (Figure 6, 'DBP2Δ'). In the wild-type strain exogenous Dbp2p is initially expressed poorly and gradually accumulates during the course of the induction (Figure 6, 'wt DBP2'). The rise in level of exogenous Dbp2p in the wild-type strain coincides with a gradual reduction in the amount of endogenous Dbp2p (data not shown), and reflects competition between transcripts from the exogenous *GAL1* promoter and the endogenous *DBP2* promoter. The most striking result is that the modest elevation in level of endogenous Dbp2p in the cDNA strain (Figure 2B, lanes 2, 3 and 4) is able to suppress completely expression of exogenous Dbp2p (Figure 6, 'cDBP2'). Thus,

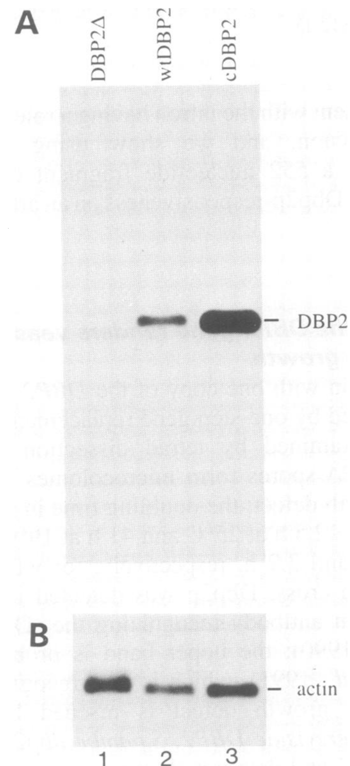


Fig. 3. Northern blot hybridized with a *DBP2* probe (**A**) and then an actin probe (**B**). Lane 1, *DBP2Δ*; lane 2, wild-type *DBP2*; lane 3, *cDBP2*. Poly-A$^+$ RNA from haploid strains with the specified allele at the endogenous *DBP2* locus was prepared from cells grown at 35°C. (Lanes 1–3: yIB12/1, 12/2 and 37, respectively)

the level of exogenous protein is inversely related to the level of endogenous protein.

The intron can confer Dbp2p-responsiveness on heterologous splicing reporters

To identify sequences which mediate Dbp2p-responsiveness, the intron and 264 bp of surrounding Dbp2p coding

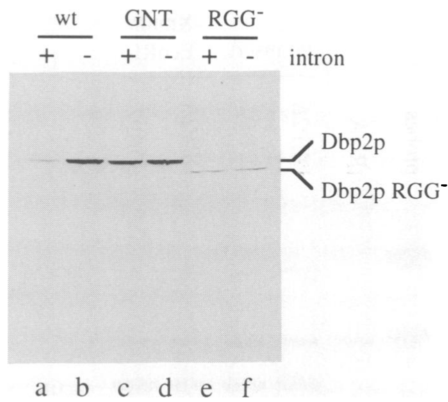


Fig. 4. Western blot probed with KT3 antibody to detect exogenous Dbp2p in a wild-type strain containing plasmids expressing *DBP2* alleles with (lanes a, c and e) or without (lanes b, d and f) the *DBP2* intron. Lanes a and b: wild-type *DBP2*; lanes c and d: GNT mutant *DBP2*; lanes e and f: RGG⁻ mutant *DBP2*. RGG⁻ Dbp2p runs faster than wild-type and GNT point-mutant protein because it lacks 49 amino acids at the C-terminus. Exogenous *DBP2* expression from the *GAL1* promoter was induced in galactose medium for 6 h. (Lanes a–f: pIG85, 83, pIB56, 58, 57 and 59, respectively in yIB12/2.)

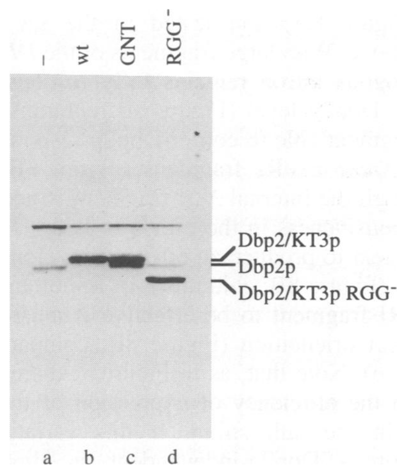


Fig. 5. Western blot probed with MaD1 showing that high level expression of active exogenous Dbp2p suppresses Dbp2p production from the endogenous intron-containing allele. Lane a is from the parental strain: the lower band is endogenous Dbp2p. Note that this band is absent in lane b but present in lanes c and d. The additional bands in lanes b–d are exogenous KT3-tagged Dbp2p: lane b, wild-type *DBP2;* lane c, GNT mutant *DBP2*; lane d, RGG⁻ mutant *DBP2*. The slower migration of exogenous Dbp2p in lanes b and c is caused by the KT3 epitope tag. Exogenous protein is produced constitutively from the *ADH1* promoter using *DBP2* alleles lacking the *DBP2* intron. (Lanes b–d: pIB71, 73 and 74, respectively in yIB12/2.)

sequence were cloned into a restriction site at codon 3 of the *lacZ* gene in a reporter plasmid (Figure 7A). This construct was then transformed into the three strains described above and Dbp2p-responsiveness was determined by measuring β-galactosidase activity. Since active Dbp2p is not produced by the reporter, the Dbp2p level remains constant throughout the assay. β-galactosidase assays show that this heterologous system remains fully Dbp2p-responsive: *lacZ* expression is high in the null strain (Figure 7B, column a) and completely suppressed in the strain overexpressing Dbp2p (Figure 7B, column c). The effect is specific because deletion of a 342 bp *Nsi*I fragment in the intron restores *lacZ* expression in the strain overexpressing Dbp2p (Figure 7B, compare columns c and f). The β-galactosidase level also rises in the wild-type strain following deletion of the *Nsi*I fragment

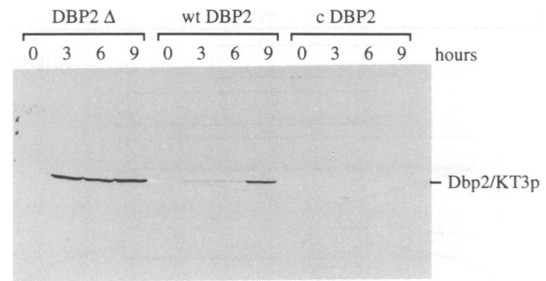
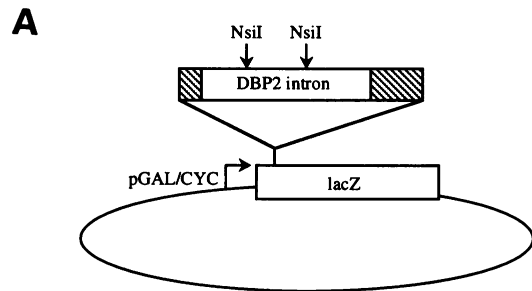


Fig. 6. Western blot probed with KT3 showing that endogenous Dbp2p can suppress exogenous *DBP2* expression. KT3-tagged wild-type Dbp2p was expressed from the *GAL1* promoter in strains with the null *(DBP2Δ)*, wild-type *(DBP2)* or intronless allele *(cDBP2)* at the chromosomal *DBP2* locus (yIB12/1, 12/2 and 37, respectively). The plasmid (pIG85) copy of *DBP2* contains the *DBP2* intron. Exogenous *DBP2* production was induced by growing cells in galactose for the indicated times.



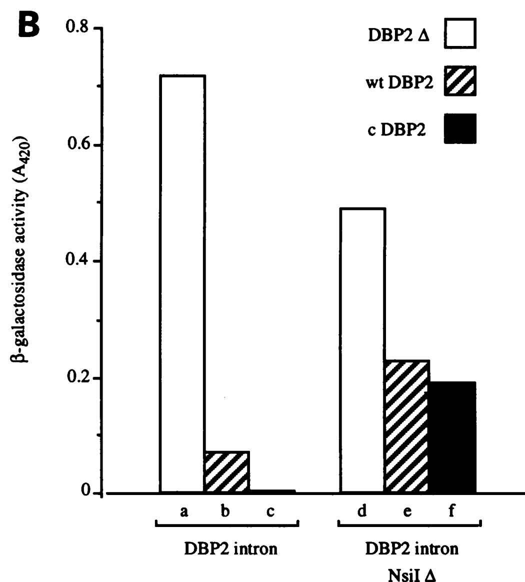


Fig. 7. The *DBP2* intron confers Dbp2p-responsiveness on a heterologous reporter. **(A)** Schematic diagram of the *lacZ* reporter constructs. The *DBP2* fragment is inserted at codon 3 of the *lacZ* reporter. **(B)** β-galactosidase assays on reporter strains containing either the complete *DBP2* intron (columns a–c) or the *Nsi*I-deleted form (columns d–f). β-galactosidase expression was measured after growing cells in galactose for 5 h. (a–c, pLS150; d–f, pLS151; a and d, yIB12/1; b and e, yIB12/2; c and f, yIB37.)

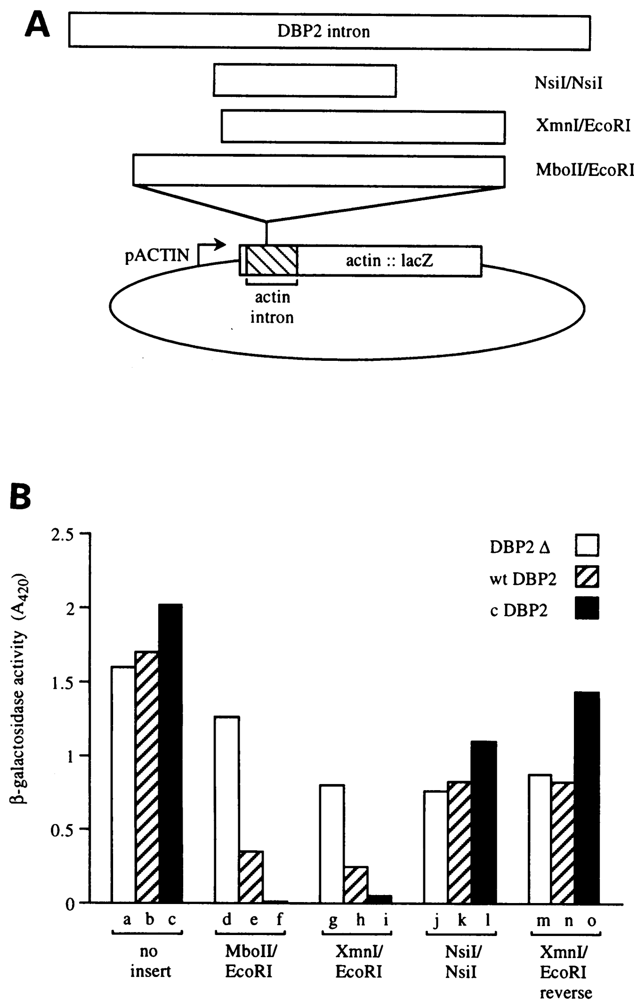


Fig. 8. Internal fragments of the *DBP2* intron confer Dbp2p-responsiveness on the actin intron. (**A**) Schematic diagram of the actin::*lacZ* reporter constructs. (**B**) β-galactosidase assays on reporter strains containing the specified *DBP2* intron fragments cloned into the *Xho*I site in the actin intron. The reporter is constitutively expressed from the actin promoter. (a, d, g, j and m, yIB12/1; b, e, h, k and n, yIB12/2; c, f, i, l and o, yIB37; a–c, DJ1; d–e, pIB21; g–i, pIB28; j–l, pIB23; m–o, pIB29.)

(Figure 7B, compare columns b and e), suggesting that even normal levels of Dbp2p may have an effect on the intron-containing reporter. However, differences of this magnitude should be interpreted with caution. Independently of Dbp2p feedback effects, differences in size and sequence may affect the efficiency of splicing of large introns in *S.cerevisiae* (for example, compare columns a and d in Figure 7B). It should also be noted that the slower growth rate of the null strain may contribute to the higher absolute β-galactosidase activity in the null relative to the other strains (Figure 7B, column d versus columns e and f). These differences are of the order of 2-fold and should be contrasted with the Dbp2p feedback effect, which leads to an increase from undetectable to normal levels of β-galactosidase activity (Figure 7B, compare columns c and f).

A 552 bp DBP2 intron fragment confers Dbp2p-responsiveness on the actin intron

To see whether the *DBP2* splice sites are necessary for Dbp2p-responsiveness, internal fragments of the *DBP2* intron were cloned into the actin intron in an actin::*lacZ* reporter (Figure 8A) and tested in the set of strains described above. With large fragments of the *DBP2* intron, the heterologous intron remains fully responsive to the endogenous Dbp2p level (Figure 8B, columns d–f). The smallest fragment able to confer Dbp2p-responsiveness is a 552 bp *Xmn*I–*Eco*RI fragment (Figure 8B, columns g–i). Although the internal *Nsi*I fragment is necessary for Dbp2p-responsiveness in the previous assay (Figure 7) it is not sufficient to produce an effect when cloned into the actin intron (Figure 8B, columns j–l). Importantly, for the *Xmn*I–*Eco*RI fragment to be effective it must be cloned in the correct orientation (Figure 8B, compare columns g–i with m–o). Note that, as in Figure 7, there is ~2-fold variation in the efficiency of expression of the different constructs in the null strain. If this variation, which by definition is Dbp2p-independent, is eliminated by normalizing the β-galactosidase activity of the individual constructs to the level in the null strain, the Dbp2p feedback effect in the cDNA strain is between 26-fold (*Xmn*I–*Eco*RI fragment in forward and reverse orientations) and 117-fold (*Mbo*II–*Eco*RI fragment versus no insert).

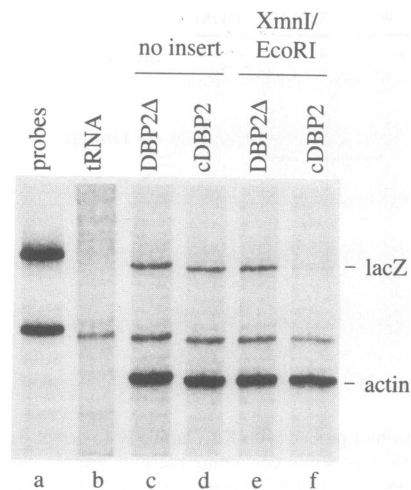


Fig. 9. Dbp2p feedback decreases heterologous reporter expression at the RNA level. RNase protection assay with a *lacZ* probe and an actin probe (loading control). Strains in lanes c–f as in Figure 8 lanes a, c, g and i, respectively.

β-galactosidase activity provides a convenient but indirect means to study feedback of Dbp2p on *DBP2* intron-containing reporters. An RNase protection assay using a *lacZ* probe was therefore performed on the null and cDNA strains containing the actin::*lacZ* reporters with and without the *Xmn*I–*Eco*RI inserts. This showed that when β-galactosidase activity is low, the *lacZ* RNA level is also low (compare Figure 9, lanes c–f with Figure 8B, columns a, c, d and f).

Feedback acts before the first step of splicing

Intron-mediated feedback could occur at several steps during the transcription and processing of the *DBP2* pre-mRNA. Despite the large size of the *DBP2* intron, which might be expected to lead to accumulation of *DBP2* pre-mRNA, the *DBP2* pre-mRNA is undetectable on Northern blots. However, regulation of the first step of splicing could be missed if the pre-mRNA were intrinsically

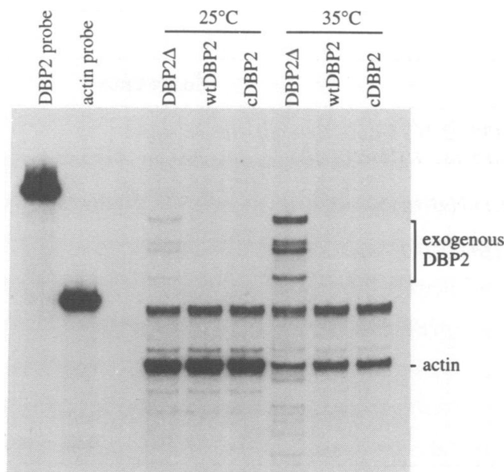


Fig. 10. Dbp2p feedback does not lead to accumulation of *DBP2* intron-containing pre-mRNA. RNase protection assay using probes for *DBP2* pre-mRNA (intron and exon 2) and actin mRNA (loading control). All of the strains contain the *prp9-1* allele (25°C, permissive; 35°C, restrictive temperature). (DBP2Δ, yIB295; wtDBP2, yIB297; cDBP2, yIB296.)

unstable. To overcome this problem, the null and cDNA alleles were crossed into a *prp9-1* background (Chapon and Legrain, 1992), which results in pre-mRNA accumulation because of a defect in the first step of splicing, and the *DBP2* pre-mRNA was expressed from the *GAL1* promoter on a plasmid. An RNase protection assay was performed with a probe which recognizes the *DBP2* intron and exon 2 (Figure 10). At the temperature permissive for the *prp9-1* mutant (25°C), faint bands protected by exogenous *DBP2* pre-mRNA are visible in the null strain and, as expected, these bands are much stronger at the restrictive temperature (35°C). Their absence in the wild-type and cDNA strains indicates that *DBP2* intron-mediated feedback can occur before the first step of splicing.

Discussion

The main conclusion from this study is that *DBP2* expression is autoregulated by a mechanism involving sequences in its own intron. This provides a possible explanation for the retention in *S.cerevisiae* of such a large intron near the 3′-end of the *DBP2* open reading frame. Although very high level expression of Dbp2p is toxic, the modest increase in endogenous Dbp2p level produced by deletion of the intron is not, and the specific advantage of autoregulating *DBP2* expression via the intron is unclear.

The fact that the strain lacking only the *DBP2* intron grows normally means that any gene within the intron must be non-essential in laboratory conditions. We have not ruled out the presence of a redundant intron-encoded gene, such as an snoRNA gene. This remains an intriguing possibility given the presence in Dbp2p of the RGG motif (a motif commonly found in nucleolar proteins), the presence in the *DBP2* intron of sno-RNA C and D boxes, the precedent that snoRNA genes are often found in the introns of RNA processing genes, the fact that many snoRNA genes in *S.cerevisiae* are non-essential, and the opportunity that could exist for co-regulation of production of protein and RNA factors involved in the same RNA processing event (Fournier and Maxwell, 1993; Sollner-Webb, 1993).

The most economical model predicts that autoregulation of *DBP2* expression exploits one of the general functions of the *DBP2* gene. This is difficult to test until the normal function of the *DBP2* gene is known. Northern blotting of the *DBP2* null strain suggests that there is a defect in splicing of the *RP28* intron, although several other introns are spliced normally (unpublished data). Since pre-mRNA secondary structure can strongly influence the efficiency with which splicing signals are used (Eperon *et al.*, 1988; Chebli *et al.*, 1989; Deshler and Rossi, 1991; Goguel *et al.*, 1993), loss of an RNA helicase could readily produce a rather selective splicing defect, and autoregulation of production of such a helicase would amount to a simple extension of its normal function.

The mechanism of *DBP2* autoregulation is not clear from our data. Transcriptional repression or pausing is possible, although it should be noted that the orientation of the intron fragment is important, and the feedback is seen with several different promoters. Our data are also consistent with an effect at an early step of pre-mRNA processing. For example, Dbp2p could use its helicase activity to alter the secondary structure of the intron and thereby expose a nuclease-sensitive site in the pre-mRNA. Iost and Dreyfus (1994) recently demonstrated that low-level expression of human p68 in *E.coli* can protect mRNA from degradation and they proposed that this could be mediated by p68 altering the mRNA structure surrounding RNase E sites. Such a model does not require a specific interaction between p68 and RNase E. In contrast, He and Jacobson (1995) recently identified *DBP2* in a two-hybrid screen for genes that interact with *UPF1*. Since *UPF1* is a gene required for degradation of mRNA containing early stop codons (nonsense-mediated mRNA decay, Leeds *et al.*, 1991), this suggests a more active role for *DBP2* in targeting specific RNAs for degradation. Early in-frame stop codons are present in many yeast pre-mRNAs, and one function of the *UPF1* system is to prevent translation of unspliced cytoplasmic pre-mRNA (He *et al.*, 1993). The 3′ position of the *DBP2* intron means that *DBP2* pre-mRNA does not contain early stop codons, and one can speculate that this unusual arrangement would free *DBP2* pre-mRNA from normal *UPF1*-dependent nonsense-mediated degradation to permit specific autoregulatory control.

Interestingly, endogenous Dbp2p does not prevent plasmid-driven expression of the intron-containing GNT and RGG$^-$ mutants (Figure 4, lanes c and e), although it does prevent expression of wild-type Dbp2p (Figure 4, lane a). This suggests either that the GNT and RGG$^-$ mutants act *in cis* or, more likely, that they are *trans*-dominant. This phenomenon is again seen with the GNT mutant in Figure 5 (the Dbp2p band is stronger in lane c than in lane a). The GNT mutant is also *trans*-dominant over endogenous Dbp2p in the heterologous reporter assays, i.e. it restores *lacZ* expression from the *Mbo*II–*Eco*RI::actin intron reporter in the cDNA strain (data not shown). That this is a highly specific effect is demonstrated by a screen we have performed for high copy suppressors of Dbp2p-mediated feedback: the cDNA strain containing the *Mbo*II–*Eco*RI::actin intron reporter was transformed with 2μ plasmids carrying a library of genomic DNA

Table I. Yeast strains used in the present studies

Strain	Genotype	Source/cross
ASZ3	**a**/α *ade2-/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/can1-100*	P.Linder
yIB12	**a**/α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/ can1-100 DBP2/dbp2Δ1::URA3*	ASZ3 + pIB3
yIB15	**a**/α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 can1-100/ can1-100 DBP2/cDBP2*	ASZ3 ± pIB8
yIB36	**a**/α *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1- his3-11,15/his3-11,15 can1-100/ can1-100 cDBP2/cDBP2*	yIB15[a]
yIB12/2	α *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 DBP2*	yIB12
yIB12/1	α *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 dbp2Δ1::URA3*	yIB12
yIB37	α *ade2-1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 cDBP2*	yIB36
Cy105	**a** *ade2 ura3-52 trp1-289 leu2-3,112 arg4 prp9-1*	P.Legrain
yIB297	*ade2 ura3 trp1 leu2-3,112 his3-11,15 DBP2 prp9-1 [pIG85]*	CY105×(yIB12/1 + pIG85)
yIB295	*ade2 ura3 trp1 leu2-3,112 his3-11,15 dbp2Δ1::URA3 prp9-1 [pIG85]*	CY105×(yIB12/1 + pIG85)
yIB296	*ade2 ura3 trp1 leu2-3,112 cDBP2 prp91* [pIG85]	CY105×(yIB37 + pIG85)

[a]yIB36 was made by crossing two haploid progeny of yIB15

fragments and blue colonies were selected. The strongest suppressor obtained encodes a fragment of the *DBP2* gene ending at amino acid 346, and expresses high levels of a truncated Dbp2p protein lacking helicase motifs IV, V and VI (unpublished data).

An important test of the *DBP2* feedback model will be whether Dbp2p can modify the structure of its own pre-mRNA, because the existing data are also consistent with an indirect action. The shortest identified fragment which confers *DBP2*-responsiveness is a 552 bp *Xmn*I–*Eco*RI fragment. RNA base pairing could readily bring the 5′ and 3′ splice sites of the *DBP2* intron into close proximity and thereby facilitate splicing, but such long range interactions are difficult to map by deletion analysis or with RNA folding programs. Phylogenetic comparison with the *Kluveromyces lactis* homologue was used to map the Rpl32p binding site (Eng and Warner, 1991) and the same approach could usefully be applied to the *DBP2* intron. Since the feedback occurs before the first step of splicing and does not specifically require the *DBP2* splice sites, it is possible that the target sequence need not be within an intron at all, a possibility we are currently testing. If true, this would greatly facilitate experiments to map the critical domains in the intron.

In addition to the feedback data, we have shown that deletion of the *DBP2* gene produces a cold-sensitive growth defect. Mutations in the *DED1*, *PRP28* and *SPB4* genes, all of which encode DEAD-box proteins, also produce cold-sensitivity (Sachs and Davis, 1990; Jamieson *et al.*, 1991; Strauss and Guthrie, 1991). Cold-sensitivity following deletion or mutation of RNA helicases could plausibly reflect the greater stability of RNA duplexes at low temperatures, and it will be interesting to see whether mutation or deletion of other DEAD-box genes has a similar effect.

Materials and methods

Plasmids and strains

Detailed descriptions of plasmids are available from I.Barta. The plasmid containing the genomic *DBP2* clone, pIG75, was obtained by gap repair in strain DJY84 (Iggo *et al.*, 1991) between the *Xba*I site at codon 18 and the *Nsi*I site 72 bp after the stop codon of the *DBP2* open reading frame. The plasmid used for the *DBP2* deletion, pIB3, contains the *URA3* gene cloned between the same sites. pIG75 and pIB3 contain 0.7 kb upstream and 1.5 kb downstream of the *DBP2* open reading frame. The *DBP2* cDNA clone was made by replacing the *DBP2* intron-containing *Xba*I–*Bgl*II fragment with a 517 bp cDNA fragment obtained from DJY84 by PCR. The plasmid used for deletion of the *DBP2* intron, pIB8, contains the *DBP2* cDNA (from the initiator methionine to the 3′-*Nsi*I site) followed by the *URA3* gene. pIG75 is derived from pFL39 (Bonneaud *et al.*, 1991); pIB3 and pIB8 are derived from Bluescript (Stratagene).

Full-length *DBP2* clones containing the KT3 epitope (Dbp2p C-terminus: ...RGRSNY PPEPET) and the 'GNT' ATPase A-box mutation (163 lysine to asparagine, AAG to AAC) were obtained by PCR mutagenesis. The 'KT3/RGG⁻'mutant was obtained by cloning an oligonucleotide into the *DBP2 Bgl*II site (Dbp2p C-terminus: ...KYDRRS PPEPET). The galactose-inducible *DBP2* expression vector is derived from P2 (*GAL1* promoter, *CYC1* terminator, *TRP1*, *CENVI*, *ARSH4*, Scharer and Iggo, 1992), and the constitutive vector is derived from pLS72 (*ADH1* promoter, *CYC1* terminator, *LEU2*, *CENVI*, *ARSH4*, Ishioka *et al.*, 1993). The following constructs were made by standard techniques from the plasmids described above ('gDNA' means the clone contains the *DBP2* intron):

pGAL1-DBP2:	cDNA	pIG70
	cDNA/KT3	pIG83
	gDNA/KT3	pIG85
	cDNA/KT3/GNT	pIB58
	gDNA/KT3/GNT	pIB56
	cDNA/KT3/RGG⁻	pIB59
	gDNA/KT3/RGG⁻	pIB57
pADH1-DBP2:	cDNA/KT3	pIB71
	cDNA/KT3/GNT	pIB73
	cDNA/KT3/RGG⁻	pIB74

The human p68 expression vector, pIB13, contains the p68 cDNA (Iggo *et al.*, 1991) cloned into P2. The *S.pombe dbp2* expression vector, pADP68 (Matviw *et al.*, 1993), was supplied by D.Young.

The *lacZ* feedback reporter plasmids were made by cloning a *Xho*II *DBP2* fragment containing the last 15 codons of exon 1, the intron and the first 72 codons of exon 2 into the *Bam*HI site at codon 3 of a *lacI::lacZ* fusion gene (Guarente and Ptashne, 1981). The fusion is expressed from a *CYC1* minimal promoter (Guarente and Mason, 1983) regulated by *GAL1* upstream activating sequences. In pLS150 a complete 1.2 kb *Xho*II fragment was used; in pLS151 an *Nsi*I-deleted 0.9 kb *Xho*II fragment was used. The plasmids also contain the *TRP1* gene and the 2μ replication origin.

The actin::*lacZ* feedback reporter plasmids contain an *Eco*RI–*Bgl*II actin fragment (containing the promoter, exon 1, the intron and part of exon 2) fused to the *lacZ* gene (Larson *et al.*, 1983). The plasmids also contain *TRP1*, *ARS1* and *CENIII*. *DBP2* intron fragments were cloned into the unique *Xho*I site in the actin intron: DJ1, parental vector; pIB21, *Mbo*II–*Eco*RI; pIB23, *Nsi*I–*Nsi*I; pIB28, *Xmn*I–*Eco*RI; and pIB29, *Xmn*I–*Eco*RI in the reverse orientation.

The yeast strains (Table I) were derived from ASZ3, a diploid of CWO4 (Banroques *et al.*, 1986) crossed with a *GAL-HO* switched isogenic strain, and Cy105 (Chapon and Legrain, 1992). Routine yeast techniques were performed as described by Guthrie and Fink (1991). Yeast were cultured on selective minimal media: SD plus adenine and

casamino acids for trp and ura selection; SC minus relevant amino acids for selection of other markers (Guthrie and Fink, 1991). For induction of the *GAL1* promoter, cells were transferred directly from 2% glucose to 2% galactose-containing media. Two-step gene replacement was done by selecting on ura$^-$ medium for integration of pIB8 linearized at codon 496 (the intron splits codon 425) followed by counterselection on medium containing 5-fluoro-orotic acid. Construction of the *cDBP2* and *DBP2*Δ strains was verified by Southern blotting.

Northern blotting and RNase protection

The Northern blots were probed with fragments corresponding to the last 150 bp of the *DBP2* open reading frame (Figure 3A) and the last 0.9 kb of the actin open reading frame (Figure 3B). *DBP2* RNA expression was quantitated by phosphorimager and normalized to actin.

RNase protection was performed as described (Promega Methods and Applications Guide, 1991) using probes transcribed with T7 RNA polymerase. The *lacZ* probe corresponds to *lacZ* codons 532–650 (*lacZ BpmI–SacI* fragment). The actin probe corresponds to actin codons 85–192 (actin *BglII* fragment). The *DBP2* probe corresponds to the last 180 nucleotides of the intron followed by *DBP2* codons 425–497 (*DBP2 EcoRI–BglII* fragment). For Figure 9, total RNA was prepared from mid-log phase cultures grown at 32°C. For Figure 10, total RNA was prepared from cultures grown at 25°C to mid-log phase in glucose minimal medium, pelleted, resuspended in galactose minimal medium, split in two and then grown for 4 h at either 25 or 35°C.

Western blotting

Cultures were grown to an OD_{600} of 0.8 and proteins were extracted as described by Yaffe and Schatz (1984). Western blots were prepared as described by Harlow and Lane (1988) and probed with KT3 (MacArthur and Walter, 1984) or MaD1 (Iggo *et al.*, 1990) monoclonal antibodies and horseradish peroxidase-conjugated secondary antibody (Figure 2B) or alkaline phosphatase-conjugated secondary antibody. The MaD1 antibody recognizes the DEAD motif in members of the p68 family and related helicases including *DED1* and *DBP1* in *S.cerevisiae* (unpublished data).

β-galactosidase assays

The β-galactosidase assays in Figures 7 and 8 were performed as described by Miller (1972) using SDS/chloroform lysis, and activity was normalized to cell number. Strains were grown at 32°C, counted with a Coulter counter, and 5×10^7 cells were incubated with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) for 30 min at 28°C. Each value is the mean of three assays. The assays were repeated at 25 and 35°C with protein extracts, normalized to total protein level and found to give the same results.

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References

Balvay,L., Libri,D. and Fiszman,M.Y. (1993) Pre-mRNA secondary structure and the regulation of splicing. *Bioessays*, **15**, 165–169.

Banroques,J., Delahodde,A. and Jacq,C. (1986) A mitochondrial RNA maturase gene transferred to the yeast nucleus can control mitochondrial splicing. *Cell*, **46**, 837–844.

Baserga,S.J., Yang,X.D. and Steitz,J.A. (1991) An intact Box C sequence in the U3 snRNA is required for binding of fibrillarin, the protein common to the major family of nucleolar snRNPs. *EMBO J.*, **10**, 2645–2651.

Bonneaud,N., Ozier,K.O., Li,G.Y., Labouesse,M., Minvielle,S.L. and Lacroute,F. (1991) A family of low and high copy replicative, integrative and single-stranded *S.cerevisiae/E.coli* shuttle vectors. *Yeast*, **7**, 609–615.

Burgess,S.M. and Guthrie,C. (1993) Beat the clock: paradigms for NTPases in the maintenance of biological fidelity. *Trends Biochem. Sci.*, **18**, 381–384.

Burgess,S., Couto,J.R. and Guthrie,C. (1990) A putative ATP binding protein influences the fidelity of branchpoint recognition in yeast splicing. *Cell*, **60**, 705–717.

Caffarelli,E., Fragapane,P., Gehring,C. and Bozzoni,I. (1987) The accumulation of mature RNA for the *Xenopus laevis* ribosomal protein L1 is controlled at the level of splicing and turnover of the precursor RNA. *EMBO J.*, **6**, 3493–3498.

Chapon,C. and Legrain,P. (1992) A novel gene, *spp91-1*, suppresses the splicing defect and the pre-mRNA nuclear export in the *prp9-1* mutant. *EMBO J.*, **11**, 3279–3288.

Chebli,K., Gattoni,R., Schmitt,P., Hildwein,G. and Stevenin,J. (1989) The 216-nucleotide intron of the E1A pre-mRNA contains a hairpin structure that permits utilization of unusually distant branch acceptors. *Mol. Cell. Biol.*, **9**, 4852–4861.

Chen,J.H. and Lin,R.J. (1990) The yeast PRP2 protein, a putative RNA-dependent ATPase, shares extensive sequence homology with two other pre-mRNA splicing factors. *Nucleic Acids Res.*, **18**, 6447.

Company,M., Arenas,J. and Abelson,J. (1991) Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from spliceosomes. *Nature*, **349**, 487–493.

Dabeva,M.D., Post,B.M. and Warner,J.R. (1986) Autogenous regulation of splicing of the transcript of a yeast ribosomal protein gene. *Proc. Natl Acad. Sci. USA*, **83**, 5854–5857.

Dalbadie,M.G. and Abelson,J. (1990) PRP5: a helicase-like protein required for mRNA splicing in yeast. *Proc. Natl Acad. Sci. USA*, **87**, 4236–4240.

Deshler,J.O. and Rossi,J.J. (1991) Unexpected point mutations activate cryptic 3′ splice sites by perturbing a natural secondary structure within a yeast intron. *Genes Dev.*, **5**, 1252–1263.

Eng,F.J. and Warner,J.R. (1991) Structural basis for the regulation of splicing of a yeast messenger RNA. *Cell*, **65**, 797–804.

Eperon,L.P., Graham,I.R., Griffiths,A.D. and Eperon,I.C. (1988) Effects of RNA secondary structure on alternative splicing of pre-mRNA: is folding limited to a region behind the transcribing RNA polymerase? *Cell*, **54**, 393–401.

Fournier,M.J. and Maxwell,E.S. (1993) The nucleolar snRNAs: catching up with the spliceosomal snRNAs. *Trends Biochem. Sci.*, **18**, 131–135.

Fragapane,P., Prislei,S., Michienzi,A., Caffarelli,E. and Bozzoni,I. (1993) A novel small nucleolar RNA (U16) is encoded inside a ribosomal protein intron and originates by processing of the pre-mRNA. *EMBO J.*, **12**, 2921–2928.

Goguel,V., Wang,Y. and Rosbash,M. (1993) Short artificial hairpins sequester splicing signals and inhibit yeast pre-mRNA splicing. *Mol. Cell. Biol.*, **13**, 6841–6848.

Gorbalenya,A.E., Koonin,E.V., Donchenko,A.P. and Blinov,V.M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.*, **17**, 4713–4730.

Guarente,L. and Mason,T. (1983) Heme regulates transcription of the *CYC1* gene of *S.cerevisiae* via an upstream activation site. *Cell*, **32**, 1279–1286.

Guarente,L. and Ptashne,M. (1981) Fusion of *E.coli lacZ* gene to the cytochrome c gene of *S.cerevisiae*. *Proc. Natl Acad. Sci. USA*, **78**, 2199–2203.

Guthrie,C. and Fink,G. (1991) Guide to yeast genetics and molecular biology. *Methods Enzymol.*, **194**.

Harlow,E. and Lane,D.P. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

He,F. and Jacobson,A. (1995) Identification of a novel component of the nonsense-mediated mRNA decay pathway using an interacting protein screen. *Genes Dev.*, **9**, 437–454.

He,F., Peltz,S.W., Donahue,J.L., Rosbash,M. and Jacobson,A. (1993) Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1-* mutant. *Proc. Natl Acad. Sci. USA*, **90**, 7034–7038.

Hirling,H., Scheffner,M., Restle,T. and Stahl,H. (1989) RNA helicase activity associated with the human p68 protein. *Nature*, **339**, 562–564.

Iggo,R.D. and Lane,D.P. (1989) Nuclear protein p68 is an RNA-dependent ATPase. *EMBO J.*, **8**, 1827–1831.

Iggo,R., Picksley,S., Southgate,J., McPheat,J. and Lane,D.P. (1990) Identification of a putative RNA helicase in *E.coli*. *Nucleic Acids Res.*, **18**, 5413–5417.

Iggo,R.D., Jamieson,D.J., MacNeill,S.A., Southgate,J., McPheat,J. and Lane,D.P. (1991) p68 RNA helicase: identification of a nucleolar form and cloning of related genes containing a conserved intron in yeasts. *Mol. Cell. Biol.*, **11**, 1326–1333.

Iost,I. and Dreyfus,M. (1994) mRNAs can be stabilised by DEAD-box proteins. *Nature*, **372**, 193–196.

Ishioka,C., Frebourg,T., Yan,Y.X., Vidal,M., Friend,S.H., Schmidt,S. and Iggo,R. (1993) Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nat. Genet.*, **5**, 124–129.

Jamieson,D.J., Rahe,B., Pringle,J. and Beggs,J.D. (1991) A suppressor

of a yeast splicing mutation *(prp8–1)* encodes a putative ATP-dependent RNA helicase. *Nature*, **349**, 715–717.

Kiledjian,M. and Kadesch,T. (1991) Post-transcriptional regulation of the human liver/bone/kidney alkaline phosphatase gene. *J. Biol. Chem.*, **266**, 4207–4213.

Larson,G.P., Itakura,K., Ito,H. and Rossi,J.J. (1983) *S.cerevisiae* actin-*E.coli lacZ* gene fusions: synthetic-oligonucleotide-mediated deletion of the 309 base pair intervening sequence in the actin gene. *Gene*, **22**, 31–39.

Leeds,P., Peltz,S.W., Jacobson,A. and Culbertson,M.R. (1991) The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. *Genes Dev.*, **5**, 2303–2314.

Linder,P., Lasko,P.F., Ashburner,M., Leroy,P., Nielsen,P.J., Nishi,K., Schnier,J. and Slonimiski,P.P. (1989) Birth of the D-E-A-D box. *Nature*, **337**, 121–122.

MacArthur,H. and Walter,G. (1984) Monoclonal antibodies specific for the carboxy terminus of Simian Virus 40 large T antigen. *J. Virol.*, **52**, 483–491.

Matviw,H., Yu,G. and Young,D. (1993) Identification and genetic analysis of *S.pombe* cDNAs that suppress deletion of *IRA1* in *S.cerevisiae*. *Gene*, **129**, 147–152.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Prislei,S., Michienzi,A., Presutti,C., Fragapane,P. and Bozzoni,I. (1993) Two different snoRNAs are encoded in introns of amphibian and human L1 ribosomal protein genes. *Nucleic Acids Res.*, **21**, 5824–5830.

Rothstein,R.J. (1991) Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.*, **194**, 281–301.

Rymond,B.C. and Rosbash,M. (1992) Yeast pre-mRNA splicing. In Jones,E.W., Pringle,J.R. and Broach,J.R. (eds), *The Molecular and Cellular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 143–192.

Sachs,A.B. and Davis,R.W. (1990) Translation initiation and ribosomal biogenesis: involvement of a putative rRNA helicase and RPL46. *Science*, **247**, 1077–1079.

Scharer,E. and Iggo,R. (1992) Mammalian p53 can function as a transcription factor in yeast. *Nucleic Acids Res.*, **20**, 1539–1545.

Schmid,S.R. and Linder,P. (1992) D-E-A-D protein family of putative RNA helicases. *Mol. Microbiol.*, **6**, 283–291.

Sollner-Webb,B. (1993) Novel intron-encoded small nucleolar RNAs. *Cell*, **75**, 403–405.

Strauss,E.J. and Guthrie,C. (1991) A cold-sensitive mRNA splicing mutant is a member of the RNA helicase gene family. *Genes Dev.*, **5**, 629–641.

Vilardell,J. and Warner,J.R. (1994) Regulation of splicing at an intermediate step in the formation of the spliceosome. *Genes Dev.*, **8**, 211–220.

Wassarman,D.A. and Steitz,J.A. (1991) RNA splicing. Alive with DEAD proteins. *Nature*, **349**, 463–464.

Yaffe,M.P. and Schatz,G. (1984) Two nuclear mutations that block mitochondrial protein import in yeast. *Proc. Natl Acad. Sci. USA*, **81**, 4819–4823.

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