Dob1p (Mtr4p) is a putative ATP-dependent RNA helicase required for the 3’ end formation of 5.8S rRNA in Saccharomyces cerevisiae

Introduction

In eukaryotes, ribosome biogenesis is a complex process that involves ~80 ribosomal proteins and four mature rRNAs. The mature 60S ribosomal subunit consists of three rRNAs (5S, 5.8S and 28S-25S) and ~45 ribosomal proteins, whereas the mature 40S ribosomal subunit is formed by one 18S rRNA molecule and ~35 ribosomal proteins (reviewed in Woolford and Warner, 1991). In all eukaryotes, rRNAs are synthesized as precursors (pre-rRNAs) that require maturation by a large number of non-ribosomal trans-acting factors. Transcription of the rDNA, processing and modifications of the pre-rRNAs, and assembly with ribosomal proteins are concomitant processes that take place primarily in a specialized subnuclear compartment termed the nucleolus (reviewed in Mélèse and Xue, 1995). Although ribosome biogenesis has been studied extensively in higher eukaryotes (reviewed in Eichler and Craig, 1994), different biochemical strategies and genetic approaches in the yeast Saccharomyces cerevisiae have provided the best characterized picture regarding the various cis-elements and trans-acting factors participating in this process (reviewed in Lafontaine and Tollervey, 1995; van Nues et al., 1995; Venema and Tollervey, 1995; Tollervey and Kiss, 1997).

In yeast, three of four rRNAs (18S, 5.8S and 25S) are produced as a single 35S precursor by RNA polymerase I, whereas the fourth rRNA (5S) is transcribed independently by RNA polymerase III as a precursor, which is extended at its 3’ end by up to 10 nucleotides (Woolford and Warner, 1991; Venema and Tollervey, 1995). In the 35S pre-rRNA, the mature rRNA sequences are separated by two internal transcribed spacers, ITS1 and ITS2, and flanked by two external transcribed spacers, 5′ ETS and 3′ ETS (see Figure 1A). During the maturation of the 35S pre-rRNA, these transcribed spacers are removed by a series of ordered endo- or exonucleolytic reactions requiring small nucleolar RNAs (snoRNAs) (reviewed in Tollervey and Kiss, 1997) and proteins as trans-acting factors (Lafontaine and Tollervey, 1995; Venema and Tollervey, 1995). Concomitantly, the pre-rRNAs are also covalently modified, mostly by 2′-O-methylation of ribose groups and conversion of uridine residues to pseudouridine (Tollervey and Kiss, 1997).

The first pre-rRNA processing steps comprise the endonucleolytic cleavages in 5′ ETS and ITS1 at sites A0, A1 and A2 that yield the 20S pre-rRNA, which subsequently is converted into the mature 18S rRNA. Cleavage at site A2 also gives rise to the 27SA2 precursor, which is processed further into the 27SB1 and 27SB2 species by two alternative pathways in ITS1 (Figure 1B). Final maturation of 5.8S and 25S rRNAs requires ITS2 removal from the 27SB species. In the current model, both the 27SB1 and 27SB2 molecules follow the same processing pathway (Figure 1B). Processing at sites C1 and C2 generates the 5′ end of the mature 25S rRNA and the 3′ end of the 7S1 and 7S2 pre-rRNAs, the precursors of the mature 5.8S rRNA. Processing of the 7S pre-rRNA to the 5.8S rRNA requires a protein complex, termed the exosome, which includes Rrp4p (Mitchell et al., 1996) and Rrp41p to Rrp44p (Mitchell et al., 1997). Recombinant Rrp4p, Rrp41p and Rrp44p are each 3′→5′ exonucleases; Rrp41p is homologous to Escherichia coli RNase PH, while Rrp44p is homologous to E.coli RNase II. The other components, Rrp42p and Rrp43p, are both members of the RNase PH/PNPase family of ribonucleases, and are therefore also predicted to be 3′→5′ exonucleases (Mian, 1997). The rrp4-1 mutation or in vivo depletion of any component of the exosome results in the accumulation of forms of the 5.8S rRNA that are 3′ extended up to site C2, the 3′ end of 7S pre-rRNA. Therefore, it has been
An RNA helicase involved in 5.8S rRNA maturation

In a genetic screen for mutants dependent on overexpression of Tif3p, the yeast homologue of the mammalian translation initiation factor elf4B (Altmann et al., 1993; Coppolecchia et al., 1993), we isolated dob1-1 (dependent on elf4B). Nucleotide sequence and disruption analyses indicated that DOB1 encodes an essential member of a subfamily of putative RNA helicases that includes Ski2p (Wisner and Wickner, 1993). During the progress of this work, DOB1 was identified independently as mtr4-1, a mutation leading to nuclear poly(A)^+ RNA accumulation (Liang et al., 1996). Analysis of the dob1-1 mutant and a Dob1p-depleted strain demonstrates that Dob1p functionally interacts with the exosome complex during pre-rRNA processing.

**Results**

**Isolation of the dob1-1 mutant as a TIF3 multicopy-dependent mutation**

We have carried out a screen to isolate mutants that are dependent on a high copy number of the TIF3 gene. For this purpose, an ade2/ura3 strain (CW04, Table I) was transformed with pDK17-TIF3, a 2μ-ADE2/URA3 plasmid bearing a wild-type TIF3 gene. The resulting strain MS04 is white, but loss of pDK17-TIF3 results in red colonies. Thus, growth of MS04 on non-selective medium leads to white/red segregating colonies. This strain was mutagenized by treatment with ethylmethane sulfonate (EMS) at a dose giving a ~70% survival rate, and cells were plated out on rich, non-selective medium (YPD). Among 15,000 surviving colonies, 31 were completely white. These colonies were restreaked twice for single colonies on YPD plates, and white, non-sectoring candidates were tested for growth on plates containing 5-fluoro-orotic acid (5-FOA). Cells carrying a functional URA3 gene are sensitive to

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**Fig. 1.** Scheme of 35S pre-rRNA processing in *S. cerevisiae*. (A) Structure and processing sites of the 35S pre-rRNA. This precursor contains the sequences for the mature 18S, 5.8S and 25S rRNAs that are separated by the two internal transcribed spacers ITS1 and ITS2. Two external transcribed spacers, the 5' ETS and the 3' ETS, are present at either end. The location of various probes (numbered 1–12) used in this study are indicated. Bars represent mature rRNA species and lines the transcribed spacer. (B) Pre-rRNA processing pathway. The 35S pre-rRNA is cleaved at site A0 by the endonuclease Rnt1p, generating the 33S pre-rRNA. This molecule subsequently is processed at sites A1 and A2, resulting in the separation of the pre-rRNAs destined for the small and large ribosomal subunits. The early pre-rRNA cleavages A0 to A2 are proposed to require a large snRNP complex, which may be assisted by the putative ATP-dependent RNA helicases Dpb4p, Fal1p, Rok1p and Rrp3p. The final maturation of the 20S precursor takes place in the cytoplasm, where an endonucleolytic cleavage at site D yields the mature 18S rRNA. The 27SA2 precursor is processed by two alternative pathways that both lead to the formation of mature 5.8S and 25S rRNAs. In the major pathway, the 27SA2 precursor is cleaved at site A3 by the RNase MRP complex. The putative ATP-dependent RNA helicase Dbp3p assists in this processing step. The 27SA3 precursor is exonucleolytically digested 5'→3' up to site B18 to yield the 27SB3 precursor, a reaction requiring the exonucleases Xrn1p and Rnt1p. A minor pathway processes the 27SA2 molecule at site B19, producing the 27SB2 pre-rRNA. While processing at site B19 is completed, the 3' end of mature 25S rRNA is generated by processing at site B2. The subsequent ITS2 processing of both 27SB species appears to be identical. Cleavage at sites C1 and C2 releases the mature 25S rRNA and the 7S pre-rRNA. The latter undergoes exonuclease-dependent 3'→5' exonuclease digestion to the 3' end of the mature 5.8S rRNA. The data presented in this study indicate that Dob1p, a putative ATP-dependent RNA helicase, assists the exosome.

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proposed that the 3' end formation of 5.8S rRNA occurs via a 3'→5' exonucleolytic processing mechanism (Mitchell et al., 1996, 1997).

In addition to the nucleases, another class of proteins predicted to function enzymatically in ribosome synthesis are the ATP-dependent RNA helicases. To date, eight putative RNA helicases have been reported to be required for normal pre-rRNA processing in yeast; Dpb4p, Fal1p, Rok1p and Rrp3p are required for 18S rRNA synthesis (O'Day et al., 1996; Kressler et al., 1997; Liang et al., 1997; Venema et al., 1997a), while Dpb3p, Dpb6p, Drs1p and Spb1p are required for the maturation of the 25S and 5.8S rRNAs (Sachs and Davis, 1990; Ripmaster et al., 1992; Weaver et al., 1997; D.Kressler, J.de la Cruz, M.Rojo and P.Linder, in preparation). RNA unwinding activities might be required to provide access to the pre-rRNA at sites of an endonucleolytic cleavage, as has been suggested for Dpb3p and Fal1p (Kressler et al., 1997; Weaver et al., 1997), or they could open secondary structures in pre-rRNA substrates that might otherwise stall or block the activity of exonucleases. In addition, many snoRNAs form extensive base-paired interactions with the pre-rRNAs (Tollervey and Kiss, 1997). Thus, it is likely that RNA helicases play roles in the association/disassociation reactions of these snoRNAs with the pre-rRNA, as has been suggested for Dbp4p and Rok1p (Liang et al., 1997; Venema et al., 1997a). Finally, extensive structural rearrangements of the pre-rRNAs and the ribosomal proteins are expected to occur during the assembly reactions, and these are likely to require RNA helicases. This may be the function of Drs1p, Dpb6p and Spb1p (Sachs and Davis, 1990; Ripmaster et al., 1992; D.Kressler, J.de la Cruz, M.Rojo and P.Linder, in preparation).
Table 1. Yeast strains used in this study

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<tr>
<th>Name</th>
<th>Genotype</th>
<th>Reference/source</th>
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*aDepending on the experimental conditions, the original YCplac33-DOB1 was replaced by other DOB1-containing plasmids.

5-FOA, therefore mutants unable to lose pDK17-TIF3 should not grow on that medium. Four such mutants were identified and transformed with a CEN-LEU2 vector (YCplac111), a 2µ-LEU2 vector (YEpplac181) and their respective constructs carrying TIF3. One mutant, MS157, regained red sectored and 5-FOA resistance when transformed with YEpplac181-TIF3 but not with YEpplac181 or YCplac111-TIF3, demonstrating that MS157 carries a TIF3 multicopy-dependent mutation that was designated dobl-1. In addition, MS157 showed a slow growth phenotype at 30°C and did not grow at 37°C. Furthermore, MS157 was sensitive to the aminoglycoside paromomycin, an inhibitor of translation, to the same extent as mutants affected in translation initiation factors (tif1-1, Δtifβ, Δtif4631, cdc33-1, cdc33-42, prt1-1; de la Cruz et al., 1997) (data not shown).

To eliminate undesired second site mutations, MS157 was back-crossed to an isogenic wild-type strain (ASZ1). The heterozygous dobl-1/DOB1 diploid was neither temperature-sensitive (ts) nor sensitive to paromomycin, indicating that dobl-1 is recessive. After analysis of 20 complete tetrads of the dobl-1/DOB1 strain, the ts and the drug sensitivity phenotypes segregated 2+/-2 and co-segregated with each other, indicating that both phenotypes were due to a single nuclear mutation. A ts and drug-sensitive haploid strain (MS157-1A, Table I), derived from three consecutive crosses with ASZ1, was used to confirm the dobl-1 phenotype. As shown in Figure 2, the growth of MS157-1A was not strictly dependent on a 2µ construct harbouring TIF3 at 30°C; however, the ts phenotype was partially suppressed at 35°C by the multicopy TIF3 plasmid. This suppression was TIF3 specific, since multicopy plasmids bearing genes encoding other yeast translation initiation factors (elf4A, elf4E and elf4AG) were not able to confer suppression (data not shown). Finally, quantitative Western blot analyses showed identical Doblp levels in the wild-type (MS04 and CW04) and the dobl-1 mutant (MS157-1A) strains, either at permissive temperature or 12 h after a shift to 37°C (data not shown).
Fig. 2. The dob1-1 mutant is dependent on a high dosage of TIF3. The strain MS157-1A (dob1-1) was transformed with either YEpplac181 (2μ), YEplac181-TIF3 (2μ-TIF3), YCplac111-TIF3 (CEN-TIF3) or the complementing plasmids pDK103 (CEN-DOB1) and YCplac111-DOB1, a subclone of pDK102 (CEN-DOB1). As a control, the isogenic wild-type strain CW04 (DOB1) was transformed with YEplac181. Transformants were grown on SD-Leu plates at 30 or 35°C for 4 days.

viable to non-viable spores, with all the viable progeny being auxotrophic for histidine and, therefore, Dob1+[sup]. When JDY1 was transformed with YCplac33-DOB1 and subsequently sporulated, complete tetrads could germinate and grow. All the haploid dob1::HIS3MX6 progeny (His+)[sup] contained the YCplac33-DOB1 plasmid (Ura+) and were unable to grow on plates containing 5-FOA (data not shown). These results demonstrated that Dob1p is essential for cell viability.

To determine whether the plasmid that complemented the dob1-1 mutation contained the DOB1 gene rather than a low copy number suppressor, MS157-1A was crossed with JDY3 YCplac33-DOB1 (a dob1::HIS3MX6 strain containing a wild-type copy of DOB1 in a CEN-URA3 plasmid). After sporulation and tetrat dissection, a 2 His+:2 His− segregation was obtained. Twenty complete tetrads were tested by selection against the plasmid on plates containing 5-FOA; two viable ts and His−-spore clones were recovered in each case. These results indicated that the gene complementing the dob1-1 mutation is genetically linked to the DOB1 locus.

During the progress of this work, DOB1 was identified in a screen for mutants that accumulate nuclear poly(A)+ RNA and was designated MTR4 (Liang et al., 1996).

Construction of a GAL::DOB1 strain
To study better the function of Dob1p, a different conditional system for phenotypic analysis was established. To this end, the DOB1 ORF was cloned into pAS24 under the control of a hybrid GAL1-10 promoter, which allows expression in medium containing galactose but is repressed in glucose-based medium. The plasmid pAS24-DOB1 fully complemented the dob1 null (JDY3) on YPGal but resulted in impaired growth on YPD plates (Figure 3A). Reproducibly, after shifting a mid-logarithmic culture of JDY3 pAS24-DOB1 from liquid YPGal medium to YPD medium, the growth rate decreased progressively to a doubling time of >10 h after 24 h in YPD, as compared with the 2 h doubling time for the isogenic control strain JDY3 YCplac111-DOB1. A concomitant depletion of Dob1p was observed by Western blotting, also as compared with JDY3 YCplac111-DOB1 (Figure 3B).

The dob1-1 and GAL::DOB1 strains are deficient in 60S ribosomal subunits
The suppression by high dosage of TIF3 and the sensitivity to paromomycin suggested that the dob1-1 mutation impaired protein synthesis. To test this hypothesis, we performed polysome profile analyses. At 30°C, when
compared with wild-type cells (Figure 4A), the *dob1-1* mutant showed a deficit of free 60S relative to free 40S ribosomal subunits, an overall decrease in 80S ribosomes (free couples and monosomes) and polysomes, and an accumulation of half-mer polysomes (Figure 4B). These features were even more pronounced when *dob1-1* cells were shifted to 37°C for 12 h (data not shown). Complementation of *dob1-1* by a CEN plasmid containing *DOB1* resulted in wild-type polysome profiles (data not shown). The presence of half-mer polysomes is indicative of a defect in the late stage of the translation initiation pathway (Foioli et al., 1991; Kang and Hershey, 1994) or the result of a reduction in the number of 60S ribosomal subunits due to mutations either in proteins of the 60S ribosomal subunit (Moritz et al., 1991; Deshmukh et al., 1993; Vilardell and Warner, 1997), or in components involved in pre-rRNA processing and 60S ribosomal subunit assembly (Ripmaster et al., 1992; Hong et al., 1997; Weaver et al., 1997). To distinguish between a role for Dob1p in translation initiation or in 60S ribosomal subunit biogenesis, the relative amounts of total 40S and 60S ribosomal subunits were quantified. An A$_{254}$ nm 60S/40S ratio of ~1.5 was obtained for the *dob1-1* mutant, while this ratio was ~2 for the isogenic wild-type strain or for the *dob1-1* mutant complemented with a CEN-*DOB1* plasmid.

When polysomes were analysed from the GAL::*DOB1* strain following transfer to YPD medium, an overall decrease in polysomes and a slight appearance of half-mer polysomes were observed during depletion of Dob1p (data not shown). Quantification of total ribosomal subunits showed a 60S to 40S ribosomal subunit imbalance, which lowered the A$_{254}$ nm 60S/40S ratio to ~1.7 after 36 h of depletion. These results indicated that the *dob1-1* mutation and the *in vivo* depletion of Dob1p both lead to similar deficiencies in the 60S ribosomal subunit synthesis.

Furthermore, Western blot analyses on ribosomal subunits purified after sucrose gradient fractionation failed to detect Dob1p (data not shown). In addition, we and others have immunolocalized an N-terminally HA-tagged Dob1p in the nucleus (Liang et al., 1996, and data not shown).

Taking all these results together, we conclude that Dob1p is not a structural component of the 60S ribosomal subunit but is required for its normal biogenesis.

**The *dob1-1* mutation impairs pre-rRNA processing**

To determine whether Dob1p is required for synthesis or processing of pre-rRNAs, *dob1-1* (MS157-1A) and *DOB1* (CW04) cells were labelled *in vivo* with [methyl-$^3$H]-methionine at 30°C for 3 and 6 min, and chased with an excess of cold methionine for 5 or 15 min. Total RNA was extracted and samples were analysed by agarose gel electrophoresis, followed by transfer to a Nylon filter and fluorography. In wild-type cells, the 35S and 32S precursors, the 27SA and 27SB pre-rRNAs and the 20S pre-rRNA were detected within the first 3 min of the pulse (Figure 5A, lane 1). After 5 min of the chase, the majority of label was in the mature 25S and 18S rRNAs (Figure 5A, lane 3). In contrast, pre-rRNA processing was slowed in *dob1-1* cells (Figure 5A, lanes 5–8). The 27S species persisted after 15 min of the chase, and a lower yield of labelled 25S rRNA was detected. In contrast, 18S rRNA was produced from its 20S precursor with kinetics and yield similar to wild-type cells (Figure 5A, lanes 4 and 8).

To exclude a defect in methylation and to monitor the processing and synthesis of small RNAs, MS157-1A and CW04 were also labelled *in vivo* with [5,6- $^3$H]uracil for 3 and 6 min, and then chased with an excess of cold uracil for 5, 10 and 30 min. The analysis of high molecular weight RNAs by agarose gel electrophoresis gave similar results to those seen with labelled methyl-methionine (data not shown). Low molecular weight RNA species were analysed by polyacrylamide gel electrophoresis (Figure 5B). Labelling of tRNAs and 5S rRNA were comparable in *dob1-1* and wild-type cells. However, the synthesis of mature 5.8S rRNA was substantially delayed in *dob1-1* cells, and a marked accumulation of a species with the gel mobility of the 7S pre-rRNA was observed (Figure 5B, lanes 8–10).

Thus, the deficit in 60S ribosomal subunit observed in the *dob1-1* strain is a consequence of impaired pre-rRNA processing, leading to a reduced synthesis of both the mature 25S and 5.8S rRNAs.

**Dob1p is required for normal pre-rRNA processing**

To define the pre-rRNA processing steps that are affected after mutation in or *in vivo* depletion of Dob1p, steady-state levels of mature rRNA and pre-rRNA intermediates were also assessed by Northern and primer extension analyses. Northern analyses of high molecular weight RNAs confirmed the results of the pulse–chase labelling experiments (Figure 6). The levels of mature 25S rRNA were slightly reduced in the *dob1-1* mutant strain (Figure 6A, lanes 2 and 4). The 35S pre-rRNA (Figure 6B–D, lanes 2 and 4) and the aberrant 23S pre-rRNA species, which extends from the 5′ end of the 35S pre-rRNA to site A$_3$ in ITS1, accumulated (Figure 6B–D, lanes 2 and
the cleavages at sites A0, A1 and A2 are only kinetically
was depleted (data not shown). As in the
dob1-1
18S rRNA (Venema and Tollervey, 1995). This, however,
loss of the 20S pre-rRNA, and therefore of the mature
rRNA and the aberrant 23S species is associated with the
indicated.

Approximately 20,000 c.p.m. were loaded in each lane. The positions
of the different pre-rRNAs and mature rRNAs and tRNAs are
(B), transferred to a Nylon membrane and visualized by fluorography.

uracil for 5, 10 and 30 min. Total RNA was extracted, separated on
3H]uracil for 3 and 6 min, and then chased with an excess of cold

B

5.8S rRNAs. (Woolford and Warner, 1991). A reduction in the synthesis
of the mature 5.8SL and 5.8S S rRNAs. The 5.8S
synthesis or processing either in the dobl-1 mutant or
after depletion of Dob1p (data not shown).

Primer extension analyses on RNA from the dobl-1 and Dob1p-depleted strain confirmed the decrease in the
steady-state levels of 5.8SL and 5.8S S rRNAs, as shown
by the stops at sites B1L and B1S when probe 8 was used
as a primer (Figure 7C, lanes 2, 4 and 7–11). Stops at
these sites were correct at the nucleotide level, as compared
as a primer (Figure 7C, lanes 2, 4 and 7–11). Stops at
these sites were correct at the nucleotide level, as compared
with the isogenic wild-type strain, indicating that the 5
end of 5.8S rRNA was processed normally in the
dob1-1
strain strongly accumu-
lates the full-length 7S pre-rRNA. The increase in the 7S
pre-rRNA levels was accompanied by a slight depletion of the mature 5.8Sl and 5.8Ss rRNAs (Figure 7A, lanes 2 and 4). A dramatic accumulation of the 7S pre-rRNA
was also seen in the GAL::DOB1 strain upon depletion of Dob1p (Figure 7B, lanes 7–11) and, in consequence, the steady-state levels of the mature 5.8Sl and 5.8Ss rRNAs decreased (Figure 7A, lanes 7–11). Northern
analysis using probe 13, which hybridizes to sequences in
the 5S rRNA, did not reveal any alteration in 5S rRNA
synthesis or processing either in the dobl-1 mutant or
after depletion of Dob1p (data not shown).

In many mutant strains, accumulation of the 35S pre-
rRNA and the aberrant 23S species is associated with the
loss of the 20S pre-rRNA, and therefore of the mature
18S rRNA (Venema and Tollervey, 1995). This, however,
is not the case in the dobl-mutant strains, indicating that the
cleavages at sites A0, A1 and A2 are only kinetically
delayed. Interestingly, several other mutants that are
defective in the synthesis of the 25S/5.8S rRNAs, i.e.
dbp3, nip7, nop2, nop4/nop77 and rrp4 (Bergès et al.,
1994; Sun and Woolford, 1994; Mitchell et al., 1996;
Weaver et al., 1997; Zanchin et al., 1997), also show an
accumulation of the 35S pre-rRNA and 23S RNA without
depletion of the 20S pre-rRNA or 18S rRNA. We conclude
that a mild kinetic delay of processing at sites A0, A1 and
A2, rather than a block to processing at these sites, is a
distinctive characteristic of mutations interfering with 25S/
5.8S rRNA synthesis. The mechanism involved is unclear,
although some form of negative feedback on the processing
machinery responsible for these cleavages is a possibility.

Analysis of low molecular weight RNA showed a strong
accumulation of a pre-rRNA species of ~300 nucleotides in
the dobl-1 strain at 30 or 37°C (Figure 7A and B, lanes 2 and 4). This species hybridized with probes in the
5′ region of ITS2 between sites E and C2 (probe 10, Figure 7B; probe 9, data not shown), failed to hybridize
with probe 11 in the 3′ region of ITS2 or with probe 7 in the
3′ region of ITS1 (data not shown) and had the same
mobility as the 7S pre-rRNA of wild-type cells (Figure
7B). We conclude that the dobl-1 strain strongly accumu-
lates the full-length 7S pre-rRNA. The increase in the 7S
pre-rRNA levels was accompanied by a slight depletion of the mature 5.8Sl and 5.8Ss rRNAs (Figure 7A, lanes 2 and 4). A dramatic accumulation of the 7S pre-rRNA
was also seen in the GAL::DOB1 strain upon depletion of Dob1p (Figure 7B, lanes 7–11) and, in consequence, the steady-state levels of the mature 5.8Sl and 5.8Ss rRNAs decreased (Figure 7A, lanes 7–11). Northern
analysis using probe 13, which hybridizes to sequences in
the 5S rRNA, did not reveal any alteration in 5S rRNA
synthesis or processing either in the dobl-1 mutant or
after depletion of Dob1p (data not shown).

Primer extension analyses on RNA from the dobl-1 and Dob1p-depleted strain confirmed the decrease in the
steady-state levels of 5.8Sl and 5.8Ss rRNAs, as shown
by the stops at sites B1L and B1S when probe 8 was used
as a primer (Figure 7C, lanes 2, 4 and 7–11). Stops at
these sites were correct at the nucleotide level, as compared
with the isogenic wild-type strain, indicating that the 5′
end of 5.8S rRNA was processed normally in the
dobl-1
mutant strains. Increases in the primer extension stops at
both B1L and B1S sites were observed when using a probe
that hybridizes to the 7S pre-rRNA (probe 10; data not
shown). Thus, these data indicated that both the 7S L and
7S S pre-rRNAs accumulated in the dobl-1 and the Dob1p-
depleted strain.

Together, these results indicate that the dobl-1 mutation and the in vivo depletion of Dob1p specifically inhibit the 3′ processing of the 7S L and 7S S pre-rRNAs, resulting in
the accumulation of these precursors and a reduced synthesis of the mature 5.8Sl and 5.8Ss rRNAs. The 5.8S
and 25S rRNAs, which are both components of the 60S ribosomal subunit, can only be utilized in a 1:1 ratio
(Woolford and Warner, 1991). A reduction in the synthesis of the 5.8S rRNA may therefore also be responsible for the reduced accumulation of the 25S rRNA.

Interestingly, another small rRNA species accumulated
strongly after depletion of Dob1p. This species hybridized
only with probes 5′ to site A0 in the 5′ ETS region (Figure
8A and B, lane 6), and migrated on polyacrylamide gels

Fig. 5. The dobl-1 mutation results in reduced synthesis of 25S and
5.8S rRNAs. (A) Strains CW04 (DOB1) and MS157-1A (dobl-1) were
grown in YPD, transferred to SD medium lacking methionine, pulse-
labelled with [methyl-3H]methionine for 5 and 15 min. (B) Strains
CW04 (DOB1) and MS157-1A (dobl-1) were grown in YPD,
transferred to SD medium lacking uracil, pulse-labelled with [5,6-
3H]uracil for 3 and 6 min, and then chased with an excess of cold
uracil for 5, 10 and 30 min. Total RNA was extracted, separated
on 1.2% agarose-formaldehyde (A) or 7% polyacrylamide–8 M urea gels
(B), transferred to a Nylon membrane and visualized by fluorography.
Approximately 20,000 c.p.m. were loaded in each lane. The positions
of the different pre-rRNAs and mature rRNAs and tRNAs are
indicated. 4). However, the levels of the 20S pre-rRNA and
the mature 18S rRNA were not clearly affected (Figure 6A
and C). In the GAL::DOB1 strain depleted of Dob1p by
growth on glucose medium, the 23S was observed, together
with the 22S and 21S pre-RNA species that extend from
sites A0 and A1, respectively, to site A2 (data not shown).
As in the dobl-1 strain, the levels of the 20S pre-rRNA
and 18S rRNA were little altered, while the 25S rRNA
was depleted (data not shown).
Effects of the \textit{dob1-1} mutation on steady-state levels of pre-rRNA and mature rRNA species. RNA was extracted from wild-type CW04 (\textit{DOB1}) and MS157-1A (\textit{dob1-1}) cells following growth in YPD at 30°C or 12 h after a shift to 37°C. Equal amounts of total RNA were resolved on a 1.2% agarose–formaldehyde gel and transferred to a Nylon membrane for Northern hybridization. The same filter was hybridized consecutively with all of the different probes used. (A) Hybridization with probes 4 and 12 (see Figure 1A for location of the probes), base pairing to sequences within the mature 18S and 25S rRNAs, respectively; (B) probe 2 in the 5′ ETS; (C) probe 5 in ITS1, between sites D and A2; (D) probe 6 in ITS1, between sites A2 and A3; (E) probe 7, 3′ to site A3; (F) probe 10 in ITS2, between sites E and C2; (G) probe 11 in ITS2, between sites C2 and C1. The positions of the different pre-rRNAs and mature rRNAs are indicated by arrows.

as a single band of approximately the same mobility as the snoRNA snR30 (~610 nucleotides). This species is likely to extend from the 5′ end of the 35S pre-rRNA to site A0 and was designated 5′-A0. We conclude that the strain depleted of Dob1p, but not the \textit{dob1-1} mutant, is defective in the degradation of the pre-rRNA spacer fragment generated by cleavage of the 35S pre-rRNA at site A0. The detection of this fragment also indicates that processing at site A0 is generated by an endonucleolytic cleavage. We assume that the \textit{dob1-1} mutation shows incomplete penetrance for this phenotype.

\textbf{Dob1p functionally interacts with Rrp4p}

The defective 5.8S rRNA maturation observed in the \textit{dob1-1} and the \textit{GAL::DOB1} strains resembles the phenotypes described for the \textit{rrp4-1} mutant (Mitchell et al., 1996) and strains genetically depleted of Rrp4p, Rrp42p, Rrp43p or Rrp44p (Mitchell et al., 1997). These strains accumulate heterogeneous 3′-extended forms of 5.8S rRNA, which extend to the 3′ end of the 7S pre-rRNA (Figure 8C, lanes 7–11) accompanied by depletion of the 5.8S (Figure 8D, lanes 7–11) and 25S rRNAs, and they also accumulate the aberrant 23S, 22S and 21S species (Mitchell et al., 1996, 1997).

To determine whether the similarities in phenotypes extend to the defect in degradation of the 5′-A0 spacer fragment, RNA from \textit{rrp4-1} and \textit{GAL::RRP4} strains was analysed by Northern hybridization using probe 2 (Figure 1A) which hybridizes to the pre-rRNA immediately 5′ to site A0. Under non-permissive conditions, both strains clearly accumulated the 5′-A0 spacer fragment (Figure 7A, lanes 7–11). Further analyses demonstrated that the 5′-A0 fragment is also accumulated after \textit{in vivo} depletion of Rrp41p, Rrp42p, Rrp43p or Rrp44p (data not shown). When a probe that hybridizes closer to site +1 was used (probe 1; Figure 1A), heterogeneous species with sizes extending up to the 5′-A0 fragment were detected in the \textit{rrp4} mutant strains, but not in the strain depleted of Dob1p (Figure 8B, lanes 6, 9 and 11). It is very likely that these bands correspond to partially degraded 5′-A0 fragment molecules. Neither the entire 5′-A0 fragment nor partially degraded forms were detectable in strains carrying mutations in the 5′-3′ exonucleases Xrn1p and Rat1p (Figure 8A and B, lanes 12–13). These two 5′-3′ exonucleases are required for the 5′ end maturation of 5.8S rRNA (Henry et al., 1994) and for the degradation of several excised pre-rRNA spacer fragments, the A0–A1, D–A2 and A2–A3 regions (Stevens et al., 1991; E.Petfalski, T.Dandekar, Y.Henry and D.Tollervey, in preparation).

These results indicated that the turnover of the 5′-A0 pre-rRNA also occurs via a 3′→5′ exonucleolytic mechanism that requires both the exosome complex and Dob1p. Heterogeneous populations of 3′-extended forms of the 5.8S rRNA and degradation intermediates of the 5′-A0 fragment are observed in mutants affecting the exosome components, but not in the Dob1p-depleted strain. This indicated that these RNAs can be partially processed or degraded in the absence of a complete, functional exosome complex but not after the loss of Dob1p function.

Genetic interactions were also analysed between Dob1p and Rrp4p. The growth of a strain carrying both the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{Effects of the \textit{dob1-1} mutation on steady-state levels of pre-rRNA and mature rRNA species. RNA was extracted from wild-type CW04 (\textit{DOB1}) and MS157-1A (\textit{dob1-1}) cells following growth in YPD at 30°C or 12 h after a shift to 37°C. Equal amounts of total RNA were resolved on a 1.2% agarose–formaldehyde gel and transferred to a Nylon membrane for Northern hybridization. The same filter was hybridized consecutively with all of the different probes used. (A) Hybridization with probes 4 and 12 (see Figure 1A for location of the probes), base pairing to sequences within the mature 18S and 25S rRNAs, respectively; (B) probe 2 in the 5′ ETS; (C) probe 5 in ITS1, between sites D and A2; (D) probe 6 in ITS1, between sites A2 and A3; (E) probe 7, 3′ to site A3; (F) probe 10 in ITS2, between sites E and C2; (G) probe 11 in ITS2, between sites C2 and C1. The positions of the different pre-rRNAs and mature rRNAs are indicated by arrows.}
\end{figure}
An RNA helicase involved in 5.8S rRNA maturation

Fig. 7. The *dob1-1* mutation and *in vivo* depletion of Dob1p inhibits the 3′ end maturation of 5.8S rRNA. RNA was extracted from wild-type CW04 (*DOB1*) and MS157-1A (*dob1-1*) cells following growth in YPD at 30°C or 12 h after a shift to 37°C. The strains JDY3 YCplac111-DOB1 (*DOB1*) and JDY3 pAS24-DOB1 (*GAL::DOB1*) were grown in YPGal and shifted to YPD for up to 36 h, and RNA was extracted from samples harvested at the indicated times. Equal amounts of total RNA were resolved on a 7% polyacrylamide–8 M urea gel and transferred to a Nylon membrane for Northern hybridization (A and B) or subjected to primer extension analysis (C).

(A) Hybridization with probe 8 (see Figure 1A for location of the probes), complementary to sequences within the mature 5.8S rRNA species. (B) Hybridization with probe 10, base pairing to sequences within the 5′/H11032 ITS2 region. (C) Primer extension analysis through the processing sites B1S and B1L using oligonucleotide 8. Arrows indicate the positions of the different pre-rRNAs and mature rRNAs, as well as the primer extension stops corresponding to the different pre- and mature rRNA species analysed.

dob1-1 and *rrp4-1* mutations was substantially poorer than the growth of either single mutant strain at 25 or 30°C (Figure 9 and data not shown). Doubling times of 1.5, 2.6, 3.1 and 5.6 h were obtained for wild-type, *rrp4-1*, *dob1-1* and *dob1-1 rrp4-1* strains, respectively, grown at 30°C in YPD medium. However, a 2μ plasmid harbouring *RRP4* was unable to suppress the ts phenotype of the *dob1-1* mutation at 35 or 37°C (data not shown). No reciprocal suppression of the ts phenotype of the *rrp4-1* mutant was observed by a 2μ plasmid carrying *DOB1* (data not shown).

The similar phenotypes observed after mutation in, or *in vivo* depletion of, Dob1p or any component of the exosome, and the synthetic enhancement of the double *dob1-1 rrp4-1* mutant would be consistent with a physical interaction between Dob1p and the exosome complex. To examine this hypothesis, a Prot-Rrp4p was immunoprecipitated from cell lysates of JDY7 (Table I) with IgG–agarose beads and immunoprecipitates were analysed by Western blot (see Materials and methods). More than 90% of total Prot-Rrp4p could be reproducibly precipitated; however, no co-immunoprecipitation of Dob1p was detected (data not shown).

We conclude that the putative RNA helicase Dob1p functionally interacts with the exosome complex both during the 3′ processing of 7S pre-rRNA and during the degradation of the excised 5′-A0 fragment. Dob1p does not, however, appear to be stably associated with the exosome complex.

An RNA helicase involved in 5.8S rRNA maturation

Fig. 8. Comparison of the steady-state levels of the 5′-A0 fragment, 7S pre-rRNA and mature 5.8S rRNA in Dob1p, Rrp4p and Xrn1p Rat1p affected strains. Yeast strains were grown in YPD as follows: lanes 1 and 2, CW04 (*DOB1*) and MS157-1A (*dob1-1*) at 30°C; lanes 3 and 4, CW04 (*DOB1*) and MS157-1A (*dob1-1*) after a 12 h shift to 37°C; lanes 5 and 6, JDY3 YCplac111-DOB1 (*DOB1*) and JDY3 pAS24-DOB1 (*GAL::DOB1*) were grown in YPGal and shifted to YPD for 36 h; lanes 7 and 8, D150 (*RRP4*) and P54 (*rrp4-1*) at 25°C; lane 9, P54 (*rrp4-1*) after a 12 h shift to 37°C; lanes 10 and 11, D150 (*RRP4*) and P79 (*GAL::RRP4*) were grown in YPGal and shifted to YPD for 36 h; lane 12, strain 966-1C (*Δxrn1 rat1-1*) at 23°C; lane 13, strain 966-1C (*Δxrn1 rat1-1*) after a 6 h shift to 37°C. RNA was extracted from exponentially growing cells. Equal amounts of total RNA were resolved on a 6% polyacrylamide–8 M urea gel and transferred to a Nylon membrane for Northern hybridization. (A) Hybridization with probe 2 (see Figure 1A for location of the probes), complementary to sequences within the 5′ ETS region. (B) Hybridization with probe 1, 3′ to site +1 in the 5′ ETS region. (C) Hybridization with probe 10, complementary to sequences within the 5′ ITS2 region. (D) Hybridization with probe 8, base pairing to sequences within the mature 5.8S rRNA species. The positions of the excised 5′-A0 fragment, 7S precursor and mature 5.8S rRNAs are indicated by horizontal arrows.
Discussion

Here we have analysed the role of Dob1p, an essential putative ATP-dependent RNA helicase, in ribosome synthesis. Polysome analyses and quantification of total ribosomal subunits in the dob1-1 and the GAL::DOB1 strain revealed a deficit in 60S ribosomal subunits, leading to the appearance of half-mer polysomes. Similar profiles have been described for mutants defective in proteins of the 60S ribosomal subunits (Moritz et al., 1991; Deshmukh et al., 1993; VilardeLL and Warner, 1997) and for mutants defective in components involved in pre-rRNA processing and 60S ribosomal subunit assembly (Ripmaster et al., 1992; Hong et al., 1997; Weaver et al., 1997). Since we could not detect Dob1p in mature 60S ribosomal subunits, we concluded that Dob1p plays a role in the biogenesis of 60S ribosomal subunits. Consistent with this conclusion, pulse–chase analyses showed that the deficit in 60S ribosomal subunits in the dob1-1 mutant is attributable to under-accumulation of the mature 25S and 5.8S rRNAs. In contrast, synthesis of the 18S and 5S rRNAs and methylation of pre-rRNAs were not affected by the dob1-1 mutation. We have isolated the dob1-1 mutation in a screen for dependence on overexpression of Ti3p. It has recently been postulated that mammalian elF4B (the homologue of yeast Ti3p) serves as a bridge between the mRNA and the 40S ribosomal subunit, due to its ability to bind both mRNA and 18S rRNA, and interact with the p170 subunit of the 40S ribosomal subunit-associated factor elF3 (Méthot et al., 1996). Thus, ribosome binding could be facilitated by overexpression of Ti3p, partially bypassing the deficit in 60S ribosomal subunits in the dob1-1 strain.

The most striking defect in dob1-1 and GAL::DOB1 strains is the strong accumulation of the 7S pre-rRNA, accompanied by reduced synthesis of the 5.8S rRNA. Since 25S and 5.8S rRNAs are both components of the 60S ribosomal subunit, reduced 5.8S rRNA synthesis is likely to account for the reduced accumulation of 25S rRNA seen by pulse–chase labelling in the dob1-1 strain or, more severely, by Northern and primer extension analyses on RNA from the Dob1p-depleted strain. Processing of the 7S pre-rRNA to the 5.8S rRNA has been reported to be inhibited in strains that carry the rrp4-1 mutation (Mitchell et al., 1996) or which are genetically depleted of Rrp4p, Rrp41p, Rrp42p, Rrp43p or Rrp44p. These five proteins form a complex termed the exosome. Recombinant Rrp4p, Rrp41p and Rrp44p each have 3′→5′ exonuclease activity, and Rrp42p and Rrp43p are each predicted to be 3′→5′ exonucleases (Mitchell et al., 1997). We conclude that the 7S pre-rRNA is processed to 5.8S rRNA by the 3′→5′ exonuclease activity of the exosome, and that this also requires Dob1p, presumably acting as an RNA helicase.

Concomitant with the depletion of Dob1p, an excised fragment of the 5′ ETS region also accumulates. This fragment extends from the 5′ end of the 35S pre-rRNA to processing site A0 (Figure 1A) and was designated 5′-A0. Strikingly, accumulation of the 5′-A0 fragment was also observed in strains carrying the rrp4-1 mutation or genetically depleted of Rrp4p, Rrp41p, Rrp42p, Rrp43p or Rrp44p. From these observations, we conclude that pre-rRNA processing at site A0 occurs by a direct endonucleolytic cleavage (see also Abou Elela et al., 1996), and that the 5′-A0 fragment released is degraded 3′→5′ by the exosome complex together with Dob1p.

In addition to the similarities of phenotypes observed in the Dob1p and exosome mutants, genetic data strongly support the close functional interaction between Dob1p and the exosome since the dob1-1 mutation is synergistically enhanced by the rrp4-1 mutation. We have, however, been unable to detect co-immunoprecipitation of Dob1p with Rrp4p. This does not exclude the possibility of a physical interaction between Dob1p and the exosome, but indicates that Dob1p is not stably associated with the complex.

The pre-rRNA substrates (7S pre-rRNA and 5′-A0 fragment) are predicted to contain a high degree of secondary structure. The proposed structure of the 5′-A0 fragment includes nine major stable stem–loop structures of variable length and stability (Yeh and Lee, 1992), while the ITS2 region is predicted to fold into a compact cloverleaf-like structure containing four stable stem–loops (Yeh and Lee, 1990; van Nues et al., 1995). Dob1p might facilitate the local unwinding of these RNA structures, allowing the exosome to associate with the pre-rRNA. Alternatively, Dob1p might open the RNA structure in front of the exosome preventing stalling of the complex. These possibilities are not, of course, mutually exclusive. In E.coli, a major pathway for mRNA decay involves the degradosome, a multi-component complex which contains the 3′→5′ exoribonuclease PNPase and the endoribonuclease RNase E, together with the putative ATP-dependent RNA helicase RhlB (Py et al., 1996). In vitro, the purified degradosome can completely degrade an RNA substrate containing a stem–loop element in the presence of ATP. In contrast, free PNPase, or the purified degradosome in the absence of ATP or after immunodepletion of RhlB, degrades this RNA only up to the stem–loop structure. These results suggested that RhlB acts by unwinding RNA secondary structures that impede the progression of the 3′→5′ exonuclease activity of PNPase (Py et al., 1996). A second example of an exoribonuclease-associated RNA helicase has come from the characterization of a 3′→5′ exonuclease complex, termed the mtEXO, found in yeast mitochondria (Margossian et al., 1996). The 3′→5′ exonuclease activity of this complex probably resides in Dss1p, which is homologous to E.coli RNase II (Dmochowska et al., 1995). Suv3p, a putative NTP-
In conclusion, we propose that Dob1p plays a broadly similar role to the putative RNA helicases associated with the degradosome and the mtEXO, but functions as a cofactor for the exosome rather than as an integral component of this complex. Dob1p is highly homologous to Ski2p, which is required for the 3′→5′ exonucleolytic degradation of cytoplasmic mRNAs following poly(A) shortening. Moreover, this process also requires Rrp41p (Ski6p), a component of the exosome (J.Jacobs Anderson et al., in preparation). This suggests that Ski2p and Dob1p have similar functions as cofactors for the exosome in the cytoplasm and the nucleus, respectively.

During the course of this work, the DOB1 gene was reported as MTR4 (Liang et al., 1996); mutations in MTR4 lead to the nuclear accumulation of poly(A)^+ RNA (Kadowaki et al., 1994; Liang et al., 1996). This accumulation has been attributed to a defect in nuclear–cytoplasmic transport of mRNA. In view of the role of Dob1p/Mtr4p in the 3′→5′ processing and degradation of pre-rRNA, an alternative explanation would be that mtr4 mutants are defective in a nuclear poly(A)^+ RNA turnover pathway. In this model, the role of Dob1p/Mtr4p would be analogous to the role of Ski2p in cytoplasmic mRNA degradation. It is notable that Dob1p is localized throughout the nucleus rather than only in the nucleolus (Liang et al., 1996, and data not shown), indicating that it does have a nucleoplasmic function. No data are available on the level of nuclear RNA turnover in yeast, but in vertebrates a very substantial fraction of the nuclear RNA population (heterogeneous nuclear RNA) is degraded in the nucleus (Brandhorst and McConkey, 1974), indicating that such a turnover pathway must exist. Future experiments should allow us to provide evidence for whether Dob1p, acting in functional association with the exosome, also plays roles in the 3′ end processing or degradation of other RNA substrates, including nuclear pre-rRNA.

Materials and methods

Strains, media and microbiological methods

The S.cerevisiae strains used in this study are listed in Table 1. Most of them are isogenic to CW04, a derivative of W303 (Bannoques et al., 1986). Genetic manipulations and preparation of standard media were according to established procedures (Ausubel et al., 1994; Kaiser et al., 1994). Antibiotic-containing plates were prepared by adding the drugs from stock solutions into YPD before pouring the plates. Effective antibiotic concentrations were usually 25 μg/ml for G418, 200 μg/ml for bleomycin and 5 μg/ml for uridine.

Plasmids

Plasmid pDK17-TIF3 was constructed by cloning the TIF3 gene as a 3.5 kb EcoRI fragment from pSEY18-STM1 (Coppolecchia et al., 1993) into the EcoRI site of pASZ12-URA3. Plasmid pASZ12-URA3 was generated by introducing a 1.1 kb BglII URA3 fragment from pFL44S (Bonneaud et al., 1991) into the BamHI site of the 2μ-ADE2 plasmid pASZ12 (Stotz and Linder, 1990). YCplac11-TIF3 (CEN-URA3), YEpplac181-TIF3 (2μ-LEU2), YEpplac181-CDC33, YEpplac181-TIF2 and YEpplac181-TIF631 have been described (de la Cruz et al., 1997). Plasmids pRS415-RRP4 (CEN-URA2), pRS415-rrp4-1, pRS425-RRP4 (2μ-LEU2) and pRS416-ProA-RRP4 (CEN-URA3) have been described previously (Mitchell et al., 1996, 1997).

Isolation of TIF3 multicopy-dependent mutants

The strain MS157-1A was transformed with a YCplac11-based yeast genetic library (D.Kressler, unpublished data), and transformants were selected on SD-Leu plates at 37°C. Eleven out of 30 000 Leu^+ transformants were able to restore wild-type growth. After plasmid rescue, only two overlapping clones (pDK102 and pDK103) were able to complement the ts phenotype reproducibly when back-transformed into MS157-1A. A 4 kb subclone from pDK102 was the minimal complementing fragment and was called YCplac11-DOB1. Plasmids YCplac33-DOB1, YEpplac195-DOB1 and YEpplac181-DOB1 were obtained by inserting a 4 kb KpnI-SphI fragment from YCplac11-DOB1 into the KpnI–SphI sites of YCplac33 (CEN-URA3), YEpplac195 (2μ-URA3) and YEpplac181 (2μ-LEU2) (Gietz and Sugino, 1988), respectively.

An almost complete dob1 deletion construct was obtained by long flanking homology region-PCR (LFH-PCR) (Wach et al., 1997). The oligonucleotide pairs MS157-5PL/MS157-P5 and MS157-3PL/MS157-P3 (Table II) were used for generating a PCR product containing the 5′ LFH and 3′ LFH, respectively, using as a template genomic DNA from CW04. These PCR products were then used as primers to amplify the heterologous HIS3MX6 marker module from the EcoRV-linearized plasmid pFA6a-HIS3MX6 (Wach et al., 1997). The final PCR products were used to transform the diploid ASZ3 strain. Five His^+ transformants were examined for a Southern analysis to confirm that integration had occurred at the DOB1 genomic locus. One disrupted strain, JDY1, was sporulated and tetrads were dissected. In addition, JDY1 was transformed with YCplac33-DOB1 and subjected to tetrat analysis. A His^+ haploid segregant (JDY3) that requires a plasmid-borne copy of DOB1 for cell viability was used in further experiments.

Construction of a GAL::DOB1 allele and in vivo depletion of Dob1p

A 5 kb SphI fragment from pDK103 containing the DOB1 ORF was cloned into the SphI site of a pUC19 vector lacking the Km^R-BamHI–Xhol restriction sites in its polylinker to generate pC132. A 0.45 kb fragment containing the 5′ end of the DOB1 ORF, was PCR amplified using the oligonucleotides J7-MS157 and J9-MS157 (Table II), digested with SalI and XbaI and cloned into those sites of pC132 to generate plasmid pJC133. Finally, a 4 kb SalI–SphI fragment of pJC133 was cloned into the SalI–SphI sites of the YCplac11-based plasmid pAS24 (Schmidt et al., 1997). The resulting plasmid, pAS24-DOB1, contains a GAL10 promoter, a start codon followed by a double HA tag, and the DOB1 ORF and its 3′ contiguous region. This construct was transformed into the strain JDY3 YCplac33-DOB1. The subsequent counter-selection of YCplac33-DOB1 on 5-FOA plates resulted in the strain JDY3 pAS24-DOB1. We also refer to this strain as GAL::DOB1 or, if grown in YPD medium, as the Dob1p-depleted strain.

For in vivo depletion of Dob1p, JFY3 pAS24-DOB1 was grown in liquid medium containing galactose (YPGal) until mid-exponential phase. Cells were harvested, washed and resuspended in medium containing glucose (YPD). Cell growth was monitored over a period of 36 h, during which the cultures were diluted regularly into fresh YPD medium in order to maintain exponential growth. As a control, JDY3 YCplac11-DOB1 was used. Samples for Western blot, polysome analyses and RNA extraction were taken after 0, 6, 12, 24 and 36 h in YPD medium.

Pulse–chase labelling experiments

Pulse-chase labelling of pre-rRNA was performed as previously described (Sachs and Davis, 1990; Tollervey et al., 1993) using 250 μCi of [methyl-^3H]methionine (Amersham, 70–85 Ci/mmol) or 50 μCi of [5,6-^3H]uracil (Amersham) by capillary blotting. After incubation for 2 h at 80°C, the...
membranes were sprayed with ENHANCE™ (Du Pont), dried and exposed to X-ray films for 4 days at –80°C with an intensifying screen. For analysis of small RNAs, 20 000 c.p.m. ([5,6-3H]uracil labelling) was added to samples after hybridization and the mixture was exposed to X-ray films at –80°C with an intensifying screen. Total RNA in Figure 8 was resolved on 7% polyacrylamide–8 M urea gels. RNA was transferred by the acid–phenol method (Ausubel et al., 1989). The membranes were then exposed to X-ray films at –80°C with an intensifying screen.

**Table II. Oligonucleotides used in this study**

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<th>Name</th>
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<td>GGCTCTCTCTCTGCGG</td>
<td>5’ ETS, 5’ to A0</td>
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PX303: GAAGGCGGATCTTAGT
MS157-5PL: GGGAAGCTCGGAGCAGCGACCTCTTGGTATGAGAAGGAGATCTATTATATTTC
MS157-3PL: AAAACGAGCTCGAAGAATCTTACGAGTATATGAGAAGGAGATCTATTATATTTC
MS157-5P: CGGGAAATTCTCGTCGGG
MS157-P5: MS157-P3
MS157-3PL: TCTTCCCAAGTCCTCTCTGCC
J4-MS157: GGGTAATCATATAGTTTACTAG
J5-MS157: CCCTCAATCTGTCGAACCCAGAG
J7-MS157: ACCCGCTGCGGATCTCTACTGCTG
J9-MS157: GTTGCAGACCTGCCTGGCACTG
J11-MS157: CCGAGTGACAGGAGGAGCGCTGGCTG
HA-DOB1-N5: AGGAGATAGCGCCCTATAGTGGATATAGGAGTACGCATCTCTGATATATATCTA
HA-DOB1-N3: TGACTATGGCTCTCTGCTATGACGTCCCGGACTATGCACTCGAGGATTCTACTGATCTGTTCG

*For location of oligonucleotides 1–12, see Figure 1A.

**Preparation of Dob1p antibodies**

A recombinant N-terminal part of Dob1p was expressed in E.coli using a T7 system. To fuse the 5’ end of the DOB1 ORF with the T7 promoter, a PCR was performed with the oligonucleotides J4-MS157 and J5-MS157, complementary to the 5’ end of the DOB1 ORF and to the region +1017 to +1031, respectively. The primer J4-MS157 carries an additional Ndel site, in-frame with the ATG in the DOB1 ORF. The primer J5-MS157 carries an additional Xhol site. The ~1.1 kb PCR product was cut with Ndel–Xhol and cloned into pET22b (+) (Novagen). The resulting pET22b-NIDOB1 plasmid contains a His tag sequence in-frame with the 3’ end of the cloned DOB1 fragment. This plasmid was used to transform E.coli M0201 cells (lost and Dreyfus, 1995), and two positive candidates were grown at 30°C in 500 ml of Luria broth medium containing 100 μg/ml ampicillin. At an OD600 nm of 0.5, production of T7 RNA polymerase was achieved by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and the cultures were then shifted to 37°C. After 5 h, cells were harvested, washed with 50 mM sodium phosphate buffer, pH 8.0, containing 500 mM NaCl and resuspended in 5 ml of the same buffer. Then, cells were broken by ultrasonic treatment using a standard procedure (Sambrook et al., 1989), and the extract was centrifuged at 8000 g for 5 min. The supernatant was mixed with 4 ml of Ni-NTA agarose (Qiagen) equilibrated in the above-mentioned buffer, and the recombinant protein was purified as described by the manufacturer. Approximately 5 mg of purified protein was dialysed against distilled water, lyophilized and resuspended in a phosphate-buffered saline solution for injection into two rabbits (Elevage Scientifique Des Dombes). Immune sera were used at a 1:5000 dilution.

**Construction of a HA-Dob1 fusion protein and co-immunoprecipitations**

To express an N-terminally HA-tagged Dob1p fusion protein from its own promoter, a fusion PCR was performed (Ho et al., 1989). Briefly, two fragments with sequence overlap were generated in a first PCR series using EcoRI-restricted YCplac111-DOB1 as a template and the oligonucleotide couples J11-MS157/HA-DOB1-N3 and HA-DOB1-N5/J9-MS157. These PCR products, together with J11-MS157 and J9-MS157, were used for the final fusion PCR. The final product was cloned as an EcoRI-Xbal fragment into the EcoRI-Xbal sites of YCplac111-DOB1. This construct (YCplac111-HA-DOB1) was transformed into JDY3 YCplac33-DOB1, and then YCplac33-DOB1 was counter-selected on 5-FOA-containing plates. The strain JDY3 YCplac111-HA-DOB1 showed wild-type growth at all temperatures tested (18, 30 and 37°C). Quantitative Western blot analyses indicated that HA-Dob1p is expressed at wild-type levels.
Miscellaneous

Cytoplasmic ribosome preparations, polynucleotide analyses and quantification of total ribosomal subunits were performed according to Foiani et al. (1993). A heat-labile method (Ausubel et al., 1994). Immunoblotting was carried out according to standard procedures and decorated by the alkaline phosphatase procedure (Sambrook et al., 1989). Preparations of yeast cells for immunofluorescence were done as described (Pringle et al., 1991; Kressler et al., 1997). For dideoxy sequencing, a T7 Sequencing™ kit (Pharmacia) was used. DNA and protein sequence comparisons were performed at the Saccharomyces Genome Database (Stanford) and at NCBI.

Acknowledgements

We thank M.Rekik for technical assistance, M.Rojo (University of Gene) for excellent help and suggestions in immunofluorescence, and E.C.Hurt (University of Heidelberg) for the kind gift of the anti-Nop1p antibodies. We are indebted to B.Dichtl and P.Mitchell (University of Edinburgh) for providing us with strains, plasmids, RNA samples and with a pre-rRNA processing scheme. We are grateful to B.Dichtl, P.Mitchell and R.Parker (University of Arizona) for communicating results prior to publication. We thank J.Venema (Vrije University, Amsterdam), R van Nuen (University of Edinburgh) and members of the laboratories of P.L., M.Collart (University of Geneva) and D.T (University of Edinburgh) for fruitful discussions. Finally, we thank J.de la Cruz, I.Iost and K.Tanner for critical reading of the manuscript. J.d.l.C. acknowledges a fellowship from the Spanish Government (Ministerio de Educacion y Ciencia) and support from Sandoz-Stiftung and Ciba-Geigy Jubilaums-Stiftung. D.T. was supported by The Wellcome Trust. Parts of this study were done in the laboratory of D.T. by J.d.l.C. who also thanks EMBO for a short term fellowship. This work was supported by a grant from the Swiss National Science Foundation to P.L. (31-43321.95).

References


An RNA helicase involved in 5.8S rRNA maturation


