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Rok1p Is a Putative RNA Helicase Required for rRNA Processing

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The synthesis of ribosomes involves many small nuclear ribonucleoprotein particles (snorNPs) as trans-acting factors. Yeast strains lacking the snoRNA, snR10, are viable but are impaired in growth and delayed in the early pre-rRNA cleavages at sites A0, A1, and A2, which lead to the synthesis of 18S rRNA. The same cleavages are inhibited by genetic depletion of the essential snoNPs protein Gar1p. Screens for mutations showing synthetic lethality with deletion of the Snr10 gene or with a temperature-sensitive gar1 allele both identified the Rok1 gene, encoding a putative, ATP-dependent RNA helicase of the DEAD-box family. The Rok1 gene is essential for viability, and depletion of Rok1p inhibits pre-rRNA processing at sites A0, A1, and A2, thereby blocking 18S rRNA synthesis. Indirect immunofluorescence by using a ProtA-Rok1p construct shows the protein to be predominantly nucleolar. These results suggest that Rok1p is required for the function of the snorNP complex carrying out the early pre-rRNA cleavage reactions.

Ribosome biogenesis in eukaryotes takes place largely in a specialized nuclear compartment, the nucleolus (reviewed in reference 43). Here, approximately 80 ribosomal proteins are associated with the four mature rRNA molecules to form the large and small ribosomal subunits. Three of the four rRNAs (18S, 5.8S, and 25–28S rNA) are produced from a single precursor (pre-rRNA), which, in addition to the mature rRNA sequences, contains two external transcribed spacers (ETS), the 5′ ETS and 3′ ETS, and two internal transcribed spacers (ITS), ITS1 and ITS2. During maturation of the pre-rRNA, the transcribed spacers are removed in a series of processing steps carried out by endonucleases and exonucleases (see references 13 and 64 for recent reviews).

In yeast, the 35S pre-rRNA is cleaved at sites A0/A1/A2, yielding the 20S pre-rRNA, which is subsequently converted into the mature 18S rRNA (Fig. 1). A large number of trans-acting factors that are required for the three early cleavages have been identified. The major class comprises the small nucleolar ribonucleoprotein particles (snorNPs), each consisting of a small RNA molecule (snoRNA) associated with a set of proteins (42). The U3, U14, snR10, or snR30 snoRNPs. In the major processing pathway, ITS1 is cleaved at site A3 by another snoNP, RNase MRP (11, 40, 41, 55). This cleavage is rapidly followed by 5′ 3′ exonuclease degradation to site B1s, generating the 275S pre-rRNA (26). This processing requires Xrn1p and Rat1p (2, 26), both of which possess 5′ 3′ exonuclease activity in vitro (36, 37, 58). Strains lacking Xrn1p also accumulate the excised spacer fragment from the 3′ end of 18S rRNA to site A2 in ITS1 (57) and are defective in mRNA degradation; deadenylated, decapped mRNAs are strongly stabilized in xrn1 mutant strains (27, 46). Less direct evidence implicates Xrn1p in a range of further cellular activities (references 26, 29, and 31 and references therein).

In addition to the nuclease, another class of proteins predicted to function enzymatically in the processing and assembly of rRNA are the RNA helicases. Many members of this large, well-conserved family of proteins have an ATP-stimulated ATPase activity in vitro, while a small number have been shown to possess the ability to unwind RNA duplexes in an ATP-dependent manner (24, 49; reviewed in references 15 and 54). This activity is believed to be the common in vivo function of the members of this protein family. Putative ATP-dependent RNA helicases are found in all organisms and are implicated in all aspects of cellular RNA metabolism (15, 54). Proteins of the RNA helicase family share a number of conserved elements involved in ATP binding, substrate binding, and RNA unwinding (15, 54). The largest group of putative RNA helicases contains a common motif, D E A D, while another group, which includes several species required for pre-mRNA splicing, contains the related sequence D E A H. Two putative RNA helicases from yeast, Dsr1p and Sbp4p, are required for the synthesis of 25S rRNA and/or assembly of the 60S ribo-
somal subunit of which it is a component (50, 52), while another helicase, Rrp3p, is required for synthesis of the 18S rRNA (49). All three proteins belong to the DEAD-box family. The ROK1 gene was identified as a multicopy suppressor of a deletion of the gene encoding the exonuclease Xrn1p and found to encode a putative RNA helicase of the DEAD-box family (56). However, the function of ROK1 was not characterized further. Here, we describe the characterization of ROK1, which we identified in screens for mutations showing synthetic lethality with mutations in the genes encoding Gar1p and the associated snoRNA, snR10. The absence of snR10 or the genetic depletion of Gar1p inhibits a specific set of pre-rRNA cleavage reactions leading to the synthesis of mature 18S rRNA, and Rok1p is required for the same pre-rRNA cleavages.

**MATERIALS AND METHODS**

**Strains and plasmids.** E. coli M1 and DH5α were used for cloning and propagation of plasmids. Yeast strains used and constructed in this study are listed in Table 1. Construction of the plasmids pSNR10 (CEN-URA3-ADE3-
SNR1), pHiS3-SNR10 (CEN-HIS3-SNR10) and pTRP1-RRP5 (CEN-TRP1-RRP5) is described in reference 65. Construction of the vectors pJPG53 (CEN-TRP1-GAR1 flanking regions) and pPG76 (CEN-TRP1-GAR1) is described in reference 20. pJG53 contains a BamHI site separating the GAR1 upstream and downstream regions, which can be used for insertion of GAR1 wild-type or mutant alleles. Vector pPG219 (CEN-TRP1-gar1-10) was produced in this way by the insertion of the BamHI cassette containing the gar1-10 mutant allele sites (see below). Vector pPG203 (CEN-TRP1-RRP5-GAR1) was constructed by insertion of the 0.4-kb SalI-SstI fragment from pG377, containing the GAR1 gene, into the Sall-NruI site of pCH124. (33). To obtain pPG208 (CEN-LYS2-GAR1), a blunt-ended Sall-MalI fragment from YDPk (8), containing the LYS2 gene, was used to replace the BglII fragment containing the URA3 marker gene in pFL38 (accession no. X70482). This plasmid was then used for the insertion of an EcoRI-Sall fragment from pG67, containing the GAR1 gene, yielding pPG208. To confer erythromycin resistance, a BstUI fragment containing the ermA gene was inserted into the Scal site of pPG208, yielding pPG223 (CEN-LYS2-ermA-GAR1). In this vector, the wild-type GAR1 gene was substituted by the BamHI cassette from pPG219, containing the gar1-10 mutant allele, to obtain pPG225 (CEN-LYS2-ermA-gar1-10).

Isolation of gar1 conditional alleles. To isolate gar1 thermosensitive alleles, a BamHI cassette containing GAR1 was cloned into the BamHI site of pBSK (+).

Random mutations were generated by PCR with low dATP and dGTP concentrations. (10). From the PCR product, a 627-bp BamHI fragment, containing GAR1, was cloned into the BamHI site of pPG53 (CEN-TRP1-GAR1 flanking regions) and transformed to E. coli. Of the approximately 60,000 transformants, 90% were found to bear the GAR1 insert. Plasmid DNA was extracted and used to transform yeast strain YO24 (Gal::gar1 [Table 1]). Yeast transformants were selected on YPD plates at 25°C and then replica plated onto YPD plates at 37°C. Strain slc 94, carrying the ROK1 gene, was digested with HpaI and SfiI, which introduces a 4.5-kb deletion encompassing the ROK1 gene. The library plasmid was transformed back to strain YO126, which carries the SGR11 cassette, and the resulting diploid was sporulated. Upon tetrad dissection, we observed a 2:2 segregation of the dphenotype, indicating that it was caused by a mutation in a single gene.

Cloning of ROK1. Strains YJ167 (Table 1) and YJ94 (YO88 [Table 1]) were transformed with a yeast genomic library by the lithium acetate method (18) and the transformants were replica plated onto 4% YPD plates to identify segregating colonies. In both screens, the library plasmid was recovered from 126 segregants. Nine plasmids recovered from YJ167 transformants carried the wild-type SNR10 gene, whereas the other three carried different overlapping fragments. Of the plasmids recovered from YO88, 10 contained either the wild-type GAR1 or ADE2 gene whereas the other 2 contained an undetermined genomic fragment. Upon determination of the sequence of the terminal regions, all library inserts contained a region from chromosome VII. The fragment common to the three YJ167 plasmids contained three complete open reading frames (ORFs); two ORFs corresponded to known genes, NPS49 and ROK1, whereas the third ORF (gene G1654) was still uncharacterized. These plasmids were transformed back to strain YJ167 and found to complement the sectoring phenotype. Three internal deletions were then constructed in one of the library plasmids, pSS5, disrupting each of the individual ORFs, and transformed to strain YJ167. The plasmids with deletions in either the NPS49 or the unknown ORF were still able to complement the sectoring phenotype of strain YJ167. The intact plasmids were retransformed to YJ167. In order to identify the unknown ORF, the library plasmid was transformed back to strain YO88 and found to complement the synthetic-lethal phenotype. A 9-merspecific probe complementary to the ROK1 gene was synthesized.

Cloning and sequencing of the rok1 alleles. The rok1-1 allele from strain YJ167 was cloned by gap repair (51). For this, the library plasmid pSS5a was digested with NcoI and Hpal, which introduces a 4.5-kb deletion encompassing the ROK1 locus. The linearized plasmid was gel purified and retransformed to strains YJ167 and YJ160, and after His+ transformants were selected. For each strain, the plasmid from four independent transformants was rescued by transformation of a yeast miniprep to E. coli. The intact plasmids were retransformed to strain YJ167, and in all cases, the constructs derived from strain YJ160 complemented the dphenotype whereas those derived from strain YJ167 did not. To clone the mutant ROK1-1 allele from strain YO88, we prepared genomic DNA from this strain as well as the parental YO126 strain. Genomic DNA (5 μg) was digested with EcoRI-HindII and separated on a 1.2% precast agarose gel. DNA ranging in size from 2.5 to 3.5 kb was isolated. The HindII fragment containing ROK1 (1.3 kb) was gel purified and cloned into pBSK (+). The resulting partial genomic library was transformed into DH5α, and approximately 2,000 transformants were screened by colony hybridization (53) with a 40-mer oligonucleotide probe complementary to the ROK1 gene. The sequences from the two ROK1 genes and the two rok1 mutant alleles were determined by

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Remark or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YJ140</td>
<td>MAT ade2 his3 leu2 trp1 ura3</td>
<td>+ pRS316</td>
</tr>
<tr>
<td>YJ156</td>
<td>MAT ade2 his3 leu2 trp1 ura3 + pTRP1-RRP5</td>
<td>snr10 sl screen starting strain</td>
</tr>
<tr>
<td>YJ160</td>
<td>MAT ade2 his3 leu2-3,112 trp1 ura3 snr10:LEU2 + pSNR10 + pTRP1-RRP5</td>
<td>snr10 sl screen starting strain</td>
</tr>
<tr>
<td>YJ167</td>
<td>MAT ade2 his3 leu2-3,112 trp1 ura3 snr10:LEU2 rok1-1 + pSNR10</td>
<td></td>
</tr>
<tr>
<td>YJ172</td>
<td>MAT ade2 his3 leu2 trp1 ura3 GAL::rok1 (URA3)</td>
<td></td>
</tr>
<tr>
<td>YJ174</td>
<td>MAT ade2 his3 leu2-3,112 trp1 ura3 snr10::LEU2 rok1-1::HIS3-GAR1 (URA3)</td>
<td></td>
</tr>
<tr>
<td>YJ175</td>
<td>MAT ade2 his3 leu2-3,112 trp1 ura3 GAL::rok1 (URA3) + pTRP1-ProtA-Rok1</td>
<td></td>
</tr>
<tr>
<td>YO24</td>
<td>MAT ade2-101 his3 ls2-801 trp1 ura3-52 GAL::ura1 (URAS)</td>
<td></td>
</tr>
<tr>
<td>YO126</td>
<td>MAT ade2 ade3 his3 ile2 leu2-3,112 trp7-1 ura3-52 gal::LEU2 + p PG203 + p PG225</td>
<td>gal11 sl screen starting strain</td>
</tr>
<tr>
<td>YO 88</td>
<td>MAT ade2 ade3 his3 ile2 leu2-3,112 trp7-1 ura3-52 gal::LEU2 rok1-2 + p PG203 + p PG225</td>
<td>gal11 sl strain sclic 94</td>
</tr>
</tbody>
</table>

**Table 1. Yeast strains used in this study**
dideoxy sequencing. For this, a 1.7-kb NcoI-Thr111I fragment from representative clones, containing the ROK1 gene, was subcloned into pBS(KS+) and entirely sequenced.

Construction of the GAL::rok1 allele. A 2.7-kb EcoRI-SacI fragment containing the ROK1 gene was subcloned from the library plasmid p5Sa into pBS(KS+), yielding pBS-ROK1. This plasmid was linearized with Ncol, present at the ATG start codon of the ROK1 gene, blunt ended with Klenow DNA polymerase, and used for insertion into a L5-bp blunt-ended HindIII-BamHI fragment from plasmid pLGD5, containing the URA3 gene and the GAL10 promoter (23). The resulting plasmid, pBS-GAL::rok1, contains the ROK1 gene fused to the GAL10 promoter at the BamHI and Ncol sites. From pBS-GAL::rok1, a linear 4.2-kb EcoRI-SacI fragment, containing the GAL::rok1 gene and 550 bp of both 5′- and 3′-flanking sequences, was gel purified and used to transform strain YJV160 (Table 1). Colonies were initially selected on SGA-ura plates and streaked on YPD and YPGal plates to check for galactose dependence. Six galactose-dependent strains were analyzed by Southern hybridization to confirm correct integration. One such strain was chosen and designated YJV172 (Table 1). The GAL::rok1 allele was also integrated into another wild-type host strain, D150, yielding strains YJ178 (Table 1).

In vivo depletion of Rok1p. Strain YJV172 (GAL::rok1) was grown in liquid SD-alu medium until mid-exponential phase. Cells were harvested by centrifugation and resuspended to an optical density at 600 nm (OD600) of 0.06 in SD-ura. Cell growth was monitored over a period of up to 50 h, during which the cultures were regularly diluted with prewarmed medium to maintain exponential growth. A 1-sa control, YJV140 transformed with pRS316 (strain YJV156) was used. For RNA isolation, cells were harvested at 0, 12, 24, 36, and 48 h after the shift to glucose-based medium.

RNA analysis. RNA isolation, Northern hybridization, and primer extension were all carried out as described previously (6, 59, 65). In all experiments, RNA samples corresponding to 0.2 OD600 unit of cells were used, except for primer extension from within the mature rRNA regions. In this case, RNA corresponding to 0.02 OD600 unit of cells was used, whereas the 3′-end-labeled primers were mixed with a 50-fold molar excess of unlabelled primer. The following oligonucleotides were used for Northern hybridization and primer extension (see Fig. 1): 5′-TCGGGTCTCTCTGCTGC-3′, 5′-CATGGCTTAATCTTTGAGAC-3′, 5′-CTCGCTTGTAGTATGCG-3′, 5′-GCTCTTTGCTCTTGCC-3′, and 5′-GGCCAGCAATTTCAAGTTA-3′.

Construction of the ProtA::rok1 allele. Plasmid pTRP1-ROK1 contains a 2.7-kb EcoRI-SacI fragment from library plasmid p55a cloned into pBS(KS+) (CEN-TRP1). A 400-bp NcoI fragment, encoding two immunoglobulin G (IgG) binding domains of Staphylococcus aureus protein A, was then cloned into the naturally occurring Ncol site at the ATG start codon of the ROK1 gene, yielding plasmid pTRP1-ProtA::rok1. Plasmids pTRP1-ROK1 and pTRP1-ProtA::rok1 were transformed to strain YJV172 (GAL::rok1) and selected on SGA-gal plates. Transformants were streaked on YPD to test the functionality of the plasmid-borne ROK1 gene (strain YJV174) and ProtA::rok1 (YJV175) genes, respectively (Table 1).

Immunofluorescence. YJV174 and YJV175 cells were grown in YPD medium to mid-exponential phase, fixed by incubation in 4% (vol/vol) formaldehyde for 1 h at room temperature, and spheroplasted. Immunofluorescence was performed by the indirect method (9, 22). Nop1p was detected with mouse monoclonal antibody MAB663 (kindly provided by J. A. Aris, University of Florida, Gainesville, Fla.) and a secondary goat anti-mouse antibody coupled to Texas Red (Jackson ImmunoResearch, West Grove, Pa.) at both a 1:200 dilution. ProtA::rok1 was detected with a rabbit anti-protein A antibody at 1:200 followed by a secondary goat anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) at 1:160 (both from Sigma). To stain nuclear DNA, 4′,6-diamidino-2-phenylindole (DAPI) was included in the final wash step at 1 μg/ml.

RESULTS Identification of ROK1 in synthetic-lethal screens. The screens for sl mutants were based on the ade2/ade3 red/white colony sectoring assay (32, 33, 65) (see Materials and Methods for details of the screens). Two starting strains were used. Strain YJV160 carries an insertional disruption of the chromosomal SNR10 gene and the wild-type SNR10 gene on an ADE3URA3 plasmid (pSSN10). Strain YO126 carries a chromosomal disruption of the GAR1 gene, the wild-type GAR1 gene on an ADE3URASSN10 plasmid (pPG203), and a sl gar1-10 allele on a LYS2 plasmid (pPG225). The growth of strains lacking a plasmid is impaired, and on nonselective YPD plates the plasmids containing the wild-type SNR10 or GAR1 genes can be lost, yielding red/white-sectoring colonies. After mutagenesis by UV irradiation, surviving colonies were screened microscopically for nonsectoring mutants. These strains were streaked on plates containing 5-F-FOA. Sl mutants are not able to lose the wild-type SNR10 or GAR1 plasmids and should be able to grow on media containing 5-F-FOA because of the presence of the URA3 gene on the plasmid. Two such strains were identified in the screen with snr10; both strains required sectoring when transformed with an SNR10 gene but not with an empty vector, demonstrating that these cells carried sl with the snr10 deletion. By the same criteria, three sl strains were identified in the screen with gar1-10.

The gene responsible for the synthetic lethality with snr10 in strain YJV167 (sl7) was cloned by complementation of the sectoring phenotype. A yeast genomic library was transformed to YJV167, and the library plasmid was recovered from 12 sectoring colonies. In nine cases, the wild-type SNR10 gene was cloned, while the three remaining plasmids contained overlapping fragments derived from a region of chromosome VII. The fragment common to the inserts contained three complete ORFs; two corresponded to known genes, NSF49 and ROK1, whereas the third ORF (G1654) was uncharacterized. In-frame deletions were made in each of the ORFs and tested for complementation. Only the deletion in ROK1 abolished complementation of the sectoring phenotype. The rok1-1 allele in sl strain YJV167 and the ROK1 gene from the parental strain YJV160 strain were cloned by gap repair and retransformation into E. coli (see Materials and Methods). When intact plasmids carrying the parental ROK1 gene were reintroduced in the sl strain YJV167, red/white sectoring was restored, whereas this was not the case for plasmids carrying the rok1-1 allele derived from YJV167. These results demonstrate that the mutation responsible for the synthetic lethality with snr10 lies within the ROK1 gene.

One of the strains identified in the sl screen with gar1-10, slc94 (Y088 [Table 1]), was crossed with the parental strain, the diploid strain was sporulated, and tetrads were dissected. For each tetrad, haploid spores were transformed with plasmid pPG219 (CEN-TRP1-gar1-10) and transformants were tested for growth on 5-FOA, colony sectoring, and thermosensitive growth. In all cases, two sectoring haploids that grew normally on 5-FOA and two nonsectoring haploids unable to grow on 5-FOA were obtained. This 2:2 segregation shows that a mutation in a single gene is responsible for the sl phenotype. Moreover, all nonsectoring haploid strains exhibited a cold-sensitive growth phenotype, indicating that the sl mutation induced in strain slc 94 confers a cold-sensitive growth phenotype. To clone the wild-type allele of this gene, strain Y088 was transformed with a genomic library and 12 sectoring colonies were selected. Among these, 10 transformants carried either the wild-type GAR1 gene or the ADE2 gene. The plasmids from the two remaining transformants were recovered and, upon retransformation into the slc 94 strain, shown to complement the sl phenotype. One of the plasmids was partially sequenced and shown to contain a fragment from chromosome VII comprising the ROK1 gene, as well as the SUA5 and PMR1 genes and an uncharacterized ORF (G1654). Subcloning showed that the ROK1 gene alone was able to restore both sectoring and growth at 13°C of the slc 94 strain. The ROK1 genes were recovered from the parental strain, Y088, and from the slc strain, YO126 (Table 1), by screening partial genomic libraries. Upon retransformation of both alleles to strain slc 94, the ROK1 gene recovered from the wild-type strain restored red/white sectoring and growth on 5-FOA containing medium, whereas this was not the case for the rok1-1 allele recovered from the sl strain. A mutation in ROK1 is therefore also sl with the gar1-10 allele. Interestingly, the rok1-2 allele is also sl with snr10, as shown by the inability to restore red/white sectoring and growth on 5-FOA containing medium.
complement the nonsectoring phenotype of the sl strain YJV167.

ROK1 is predicted to encode a DEAD-box, ATP-dependent RNA helicase of 564 amino acids (64 kDa). The protein is basic (pI = 9.2) and contains a high percentage of hydrophilic amino acids. In addition to all of the conserved helicase motifs present in the central domain, Rok1p contains N- and C-terminal domains of approximately 160 and 80 residues, respectively. These regions, which are distinct from those in other RNA helicases, are presumed to confer unique functions to the different DEAD-box proteins. The Rok1p sl alleles were cloned into pBS(KS+) and entirely sequenced. The rok1-1 (Leu7 to Ser) and rok1-2 (Cys352 to Arg) sl alleles are also indicated.

A search in the database resulted in the identification of two overlapping expressed sequence tags (ESTs) from humans, which show homology to a region in the C-terminal part of Rok1p. Part of the homology resides in the central helicase domain, but the conservation extends into the unique, C-terminal domain (Fig. 2). The ROK1 gene had previously been cloned as a multicopy suppressor of a deletion of the GAR1 gene and was shown to be essential for cell viability (56). The mutations identified in the EST2 (accession no. N30537, respectively) are shown. Stop codons are represented by dark shading. Both ESTs probably contain frameshifts in their C-terminal region with two overlapping human ESTs (EST1 and EST2; accession nos. N30525 and N30537), respectively. The mutations identified in the EST2 (Leu7 to Ser) and EST1 (Ser to Thr) are indicated.

The level of 18S rRNA remains constant until 12 h after transfer, consistent with the gradual inhibition of Rok1p synthesis. At later time points, the level of 18S rRNA is distinguishable on galactose medium. Following transfer to glucose medium, both strains had an initial doubling time of approximately 1.7 h. The wild-type control strain maintained this growth rate, while growth of the ROK1 deletion mutant, YJV172, was used for subsequent experiments (Table 1).

To analyze the effect of Rok1p depletion on rRNA synthesis, total RNA was isolated from the GAL::rok1 strains at various time points after the shift to glucose and analyzed by Northern hybridization (Fig. 4). Hybridization with oligonucleotides complementary to the mature rRNAs shows that depletion of Rok1p does not affect the level of 25S rRNA (Fig. 4A). The level of 18S rRNA remains constant until 12 h after transfer to glucose, consistent with the absence of any growth defect up to that time. At later time points, the level of 18S rRNA is processing, suggested that Rok1p might also function in this activity.

Genetic depletion of Rok1p inhibits the synthesis of 18S rRNA. Since ROK1 had been shown to be essential for cell viability (56), we analyzed the effect of depletion of the Rok1p protein by using a conditional-lethal mutant. The ROK1 gene was placed under control of the GAL1 promoter inserted at the NcoI site, which occurs naturally at the ATG start codon (Fig. 3) (see Materials and Methods). The GAL::rok1 gene was integrated at the ROK1 genomic locus by transformation into the wild-type strain YJV140 (Table 1) and integrated into the ROK1 locus. Galactose-dependent transformants were verified by Southern analysis (data not shown). (B) Growth of the GAL::rok1 strain YJV172 (solid symbols) and its wild-type isogenic counterpart YJV156 (open symbols) after transfer from galactose- to glucose-based medium at t = 0 h. The cell density was measured at regular intervals, and the cultures were periodically diluted to maintain exponential growth.
severely reduced (Fig. 4A, compare lanes 1 to 4 and lanes 5 to 7). These data show that Rok1p is required for accumulation of 18S rRNA but not of 25S rRNA.

Rok1p is required for processing at A₀, A₁, and A₂. The effects of depletion of Rok1p on processing of the pre-rRNAs were analyzed by Northern hybridization with oligonucleotides complementary to different regions of the pre-rRNA operon (see Fig. 1A for their location). As a control, RNA prepared from the ROK1 strain (YJV156) at 0 and 48 h after transfer to glucose was used (lanes 1 and 2). The panels represent consecutive hybridizations of the same filter with the different probes. (A) Oligonucleotides 2 and 3, complementary to sites within the mature 18S and 25S rRNA sequences, respectively. (B) Oligonucleotide 1 in the 5′ ETS. (C) Oligonucleotide 4 in ITS1 upstream of site A₂. (D) Oligonucleotide 5 in ITS1 between sites A₂ and A₃. (E) Oligonucleotide 6 in ITS1 downstream of site A₃. (F) Oligonucleotide 7 in ITS2. The positions of the different pre-rRNAs are indicated by arrows.

Figure 4. Effect of Rok1p depletion on pre-rRNA processing. RNA was extracted from the GAL::rok1 strain (YJV172) at t = 0, 12, 24, 36, and 48 h after transfer to glucose (lanes 3 to 7) and analyzed by Northern hybridization with oligonucleotides complementary to different regions of the pre-rRNA operon (see Fig. 1A for their location). As a control, RNA prepared from the ROK1 strain (YJV156) at 0 and 48 h after transfer to glucose was used (lanes 1 and 2). The panels represent consecutive hybridizations of the same filter with the different probes. (A) Oligonucleotides 2 and 3, complementary to sites within the mature 18S and 25S rRNA sequences, respectively. (B) Oligonucleotide 1 in the 5′ ETS. (C) Oligonucleotide 4 in ITS1 upstream of site A₂. (D) Oligonucleotide 5 in ITS1 between sites A₂ and A₃. (E) Oligonucleotide 6 in ITS1 downstream of site A₃. (F) Oligonucleotide 7 in ITS2. The positions of the different pre-rRNAs are indicated by arrows.
The primer extension stops at sites A3, B1L, and B1S, respectively, are also unaltered during Rok1p depletion (Fig. 5B). Consistent with the results of Northern hybridization, the level of 27SA2, shown by the primer extension stop at site A2, is strongly reduced 24 h after transfer to glucose medium (Fig. 5B). Processing at sites A0, A1, and A2, respectively, are also unaltered during Rok1p depletion (Fig. 5B).

Upon depletion of Rok1p, splicing of the actin (ACT1) pre-mRNA is not detectably inhibited, as shown by Northern hybridization with the actin intron as a probe (data not shown), indicating that Rok1p is not required for pre-mRNA splicing. In addition, several snRNA species tested, U3, U14, U24, snR10, and snR30, accumulate to normal levels in the absence of Rok1p (data not shown).

Taken together, the results indicate that depletion of Rok1p leads to a specific inhibition of processing at the sites required for synthesis of the 18S rRNA, A0, A1, and A2. Subsequent processing reactions in ITS1 and ITS2 which lead to synthesis of the 5.8S and 25S rRNAs do not require Rok1p. This phenotype is similar to that observed following genetic depletion of several different snRNA species (64), but Rok1p is not required for accumulation of these snoRNAs.

**Rok1p is localized predominantly in the nucleolus.** To localize the Rok1p protein within the cell, an epitope-tagged fusion protein was constructed. An NcoI fragment containing two IgG-binding sites of S. aureus protein A was inserted in the NcoI site at the ATG start of the ROK1 gene and cloned into the episomal vector pRS314 (CEN-TRP1), yielding pTRP1-Prota::rok1. As a control, a plasmid containing the nontagged gene, pTRP1-ROK1, was used. Both plasmids were transformed into the GAL::rok1 strain, YJV172, and tested for their ability to support growth on glucose medium. The growth rates of cells expressing either the ROK1 gene or the ProtA::rok1 gene were identical, demonstrating that ProtA-Rok1p is fully functional (data not shown). Western analysis with peroxidase-coupled rabbit IgG detected a single protein with an estimated molecular mass of 75 kDa in a whole-cell lysate from the ProtA::rok1 strain (YJV175) but not from the ROK1 strain (YJV174) (Fig. 6).

ProtA-Rok1p was immunoprecipitated from cell lysates with IgG-agarose beads, and bound proteins and RNAs were recovered (see Materials and Methods). Western blotting confirmed that ProtA-Rok1p was effectively precipitated although never with 100% efficiency (data not shown). Northern hybridization did not reveal detectable coprecipitation of the snoRNAs snR10, snR30, U3, or U14 (data not shown). Total coprecipitated RNAs were 3' labeled with [5'-32P]pCp and RNA ligase. No RNA was clearly enriched in the immunoprecipitate (data not shown). While masking of the ProtA epitope in the snoRNPs remains a formal possibility, it is probable that Rok1p is not stably associated with the snoRNPs.

Indirect immunofluorescence was performed with the strains expressing either ProtA-Rok1p or Rok1p following growth in glucose medium, by using a rabbit anti-protein A antibody. As a control, Nop1p was decorated with mouse monoclonal antibody Mab66 (3). To localize the nucleus, DNA was stained with DAPI (Fig. 7, right panels). With anti-Nop1p, both strains show the typical crescent-shaped staining pattern corresponding to the different pre-rRNA cleavage sites are indicated.

**FIG. 5.** Primer extension analysis of pre-rRNA from a Rok1p-depleted strain. The samples and lane order are as in Fig. 4. (A) Primer extension with oligonucleotide 2, priming within the mature 18S rRNA region. (B) Primer extension from oligonucleotide 7 within ITS2. The positions of primer extension stops corresponding to the different pre-rRNA cleavage sites are indicated.

**FIG. 6.** Western blotting of ProtA-Rok1p. Whole-cell lysates were prepared from strains expressing Rok1p (YJV174 [lane 2]) or ProtA-Rok1p (YJV175 [lane 1]) and analyzed by Western blotting with rabbit peroxidase anti-peroxidase complex and an enhanced chemiluminescence detection kit. The positions of molecular mass markers (in kilodaltons) are indicated on the right, and the position of the ProtA-Rok1p protein is indicated on the left.
characteristic of nucleolar proteins (Fig. 7, left panels). With the anti-ProtA antibody, the ROK1 strain shows only a very low background staining whereas the ProtA::rok1 strain exhibits a nuclear signal (Fig. 7, middle panels) with nucleolar enrichment. We did not detect clear cytoplasmic staining with anti-ProtA in the ProtA::rok1 cells. The predominant localization of ProtA-Rok1p in the nucleolus is in agreement with the prominent role of Rok1p in pre-rRNA processing.

**DISCUSSION**

We have performed screens for synthetic lethality with a deletion of the nonessential snoRNA, snR10, and with a mutant allele of the gene encoding the snoRNP protein, Gar1p. Both screens identified the same gene, ROK1, that encodes a putative, ATP-dependent RNA helicase of the DEAD-box protein family. Analysis of the effects of Rok1p depletion showed that, like snR10 and Gar1p, the protein functions in the synthesis of 18S rRNA. In the absence of Rok1p, the cleavages at A₀, A₁, and A₂ are inhibited and the products of these processing steps, the 32S, 27SA₂, and 20S pre-rRNA, are lost (Fig. 4). Direct cleavage of the 35S pre-rRNA at site A₀ within ITS1 generates the aberrant 23S pre-rRNA as the 5’-terminal product. This intermediate is not further processed to mature 18S rRNA and is rapidly degraded. The 3’-terminal product, 27SA₂ pre-rRNA, is a normal processing intermediate and is converted to the mature 5.8S and 25S rRNAs in the mutant strain. This phenotype is similar to that observed following genetic depletion of components of the snR10, snR30, U3, and U14 snoRNP (21, 28, 30, 44, 61). In strains depleted of Gar1p or the U14 or snR10 snoRNPs, processing at site A₀ appears to be less inhibited than is processing at sites A₁ or A₂ (5, 64), and this is also the case for Rok1p. The accumulation of the 35S pre-rRNA and the aberrant 23S rRNA in all of these mutants indicates that cleavage at
A3 is, however, kinetically delayed (further discussed in references 34 and 64).

Processing at A0, A1, and A2 has been suggested to take place in a large complex containing the U3, U14, snR10, and snR30 snoRNAs (45). Gar1p is a component of the snR10 and snR30 snoRNPs (4, 21) and would therefore also participate in the putative complex. The similarities in the phenotypes observed upon depletion of Gar1p and snR30 suggest that inactivation of the snR30 snoRNP gives rise to the observed phenotypes in both cases (21, 44). Loss of the snR10 snoRNP gives a similar but weaker phenotype (59), and the effects of Gar1p depletion on its activity cannot therefore be readily assessed. Since mutations in ROK1 are still with mutations in either SNR10 or GAR1, it is possible that rok1 mutations are still with the loss of function of the snR10 snoRNP. This is supported by the fact that the rok1-2 mutation, identified in the sl screen with gar1-10, is also still with deletion of SNR10 in strain YJV167. It is also interesting that the gar1-10 allele codes for a protein that is truncated at position 158, thereby deleting the glycine-arginine-rich (GAR) domain of Gar1p (see also references 20 and 21). In the nucleolar protein nucleolin, this structure contains ATP-stimulated RNA-unfolding activity similar to that exhibited by some DEAD-box proteins (17, 63). The synthetic lethality between the rok1-2 and gar1-10 alleles might therefore be explained by cumulative defects in RNA unwinding reactions taking place during pre-rRNA processing and ribosome assembly.

The U3 and U14 snoRNAs bind to the pre-rRNA at sites within the 5′ETS and the 18S rRNA region, respectively, through extended base pairing (5, 7, 39) which appears to be too long for ready dissociation in the absence of a helicase activity. In contrast, snR10 and snR30 lack sequences with extended complementarity to the pre-rRNA, but it remains possible that a helicase activity associated with Rok1p is required for their association with, or dissociation from, the pre-rRNA. In comparison, the involvement of DEAD/H-box proteins in pre-mRNA splicing has been well established, although it appears that most of these proteins interact only transiently with the spliceosome (reviewed in reference 15). For none of these proteins has it been demonstrated that they actually exhibit an RNA helicase activity, but the majority have been shown to possess RNA-stimulated ATPase activity in vitro. Recently, the latter activity has been demonstrated for a human protein which appears to be intrinsic to the U5 snoRNP (35). The fact that we have been unable to detect communoprecipitation of any snoRNA (or other RNA species) with ProtA-Rok1p therefore does not exclude the possibility that Rok1p physically interacts with one or more of the snoRNPs required for 18S rRNA synthesis. Rok1p is not simply required for the synthesis of the snoRNAs, since the accumulation of all species tested, including snR10 and snR30, was unaffected by Rok1p depletion.

A part from pre-rRNA processing, ribosome biogenesis comprises rRNA modification reactions and assembly with the approximately 80 ribosomal proteins. Extensive structural rearrangements are expected to occur during all of these reactions, which are likely to require trans-acting factors. Strains lacking snR10 are impaired in growth, but the physical basis of this is unclear (59, 60). Pre-rRNA processing is clearly delayed in the mutant, but overall synthesis of the mature 18S rRNA is not reduced, and no difference in the accumulation of 18S and 25S rRNA is observed. Moreover, the growth of snR10 mutant strains is partially cold sensitive, whereas the pre-rRNA-processing defect is entirely nonconditional. Together, these observations suggest that the impaired growth of snR10 mutant strains may be due to a defect in ribosome assembly or rRNA modification rather than to the observed pre-rRNA-processing defect. M any mutants of E. coli that are defective in ribosome assembly are cold sensitive (12, 14, 25), and the cold sensitive phenotypes associated with the snR10 and rok1-2 mutations would therefore be consistent with a defect in ribosomal assembly. A n RNA helicase activity associated with Rok1p might also be required during ribosome assembly or modification, with the impaired pre-rRNA processing appearing as a consequence of this.

It is notable that several other putative RNA helicases have been shown to be involved in ribosome assembly in both E. coli and yeast. The bacterial SrmB and DsaD proteins can suppress mutations which cause defective assembly of the large and small ribosomal subunit, respectively (48, 62), while the ATPase activity of DhpA is specifically stimulated by a fragment of the bacterial 23S rRNA containing the peptidyltransferase center (16, 47). In yeast, the putative helicases Spb4p (52) and Dsr1p (50) are required for assembly of the 60S ribosomal subunit and synthesis of the 25S rRNA, while Rrp3p is required for the synthesis of yeast 18S rRNA (49). Genetic depletion of Rrp3p also inhibits pre-rRNA processing at sites A0, A1, and A2, but although depletion of Rok1p or Rrp3p gives a similar phenotype, it is clear from the genetic results that they have distinct functions. Thus, yeast ribosome synthesis requires at least four DEAD-box proteins, each with its unique function, and it is likely that more members of this protein class will be identified.

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