MOLECULAR AND CELLULAR BIOLOGY, Apr. 1993, p. 2469–2477
0270-7306/93/042469-09$02.00/0

Vol. 13, No. 4

Yeast snR30 Is a Small Nucleolar RNA Required for 18S rRNA Synthesis

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Received 11 May 1992/Returned for modification 5 August 1992/Accepted 28 January 1993

Subnuclear fractionation and coprecipitation by antibodies against the nucleolar protein NOP1 demonstrate that the essential *Saccharomyces cerevisiae* RNA snR30 is localized to the nucleolus. By using aminomethyl trimethyl-psoralen, snR30 can be cross-linked in vivo to 35S pre-rRNA. To determine whether snR30 has a role in rRNA processing, a conditional allele was constructed by replacing the authentic *SNR30* promoter with the *GAL10* promoter. Repression of snR30 synthesis results in a rapid depletion of snR30 and a progressive increase in cell doubling time. rRNA processing is disrupted during the depletion of snR30; mature 18S rRNA and its 20S precursor underaccumulate, and an aberrant 23S pre-rRNA intermediate can be detected. Initial results indicate that this 23S pre-rRNA is the same as the species detected on depletion of the small nucleolar RNA-associated proteins NOP1 and GAR1 and in an *snr10* mutant strain. It was found that the 3′ end of 23S pre-rRNA is located in the 3′ region of ITS1 between cleavage sites A2 and B1 and not, as previously suggested, at the B1 site. snR30 is the fourth small nucleolar RNA shown to play a role in rRNA processing.

In eukaryotes, the nucleolus is the center of ribosome biogenesis; imported ribosomal proteins associate with rRNAs, and the mature ribosomal subunits are then exported to the cytoplasm. The 18S, 5.8S, and 25S rRNAs are transcribed as a single transcript, the 35S pre-rRNA, which is processed via a number of intermediates (pre-rRNAs) to the mature rRNAs. It is thought that many of the ribosomal proteins assemble with the pre-rRNA while it is being processed.

There is a class of small ribonucleoprotein particles localized to the nucleolus (small nucleolar ribonucleoproteins [snoRNPs]). To date, 10 small nucleolar RNAs (snoRNAs) have been cloned from *Saccharomyces cerevisiae* (reviewed in reference 31). Seven of these snoRNAs are nonessential, and strains carrying null alleles grow normally. snR10 is not essential, but strains carrying a null allele have a cold-sensitive phenotype, and two snoRNAs, U3 and U14 (previously called snR128), are essential for growth.

U3 is the most studied of the snoRNAs. Two recent studies have examined its role in rRNA processing in *S. cerevisiae*. Hughes and Ares (11) constructed a conditional allele of U3 by placing it under the control of a regulated *GAL* promoter. It was found that depletion of U3 inhibited production of mature 18S and its 20S precursor but 25S and 5.8S continued to be synthesized normally. The processing event, A0, in the 5′ external transcribed spacer (ETS) was also abolished by U3 depletion. Beltrame and Tollervey (2) used psoralen cross-linking techniques to map the interaction between U3 and the pre-rRNA. U3 could be cross-linked in vivo to two sites in the ETS of the 35S pre-rRNA. These sites were subsequently analyzed by deletion mutagenesis; deletion of the upstream cross-linking site (+470) gave a phenotype similar to that of U3 depletion, with the loss of synthesis of 18S rRNA, but deletion of the downstream (+655) site did not affect synthesis of 18S rRNA. U3 in higher eukaryotes has also been studied. The first cleavage event in mammalian pre-rRNA processing occurs in the ETS (15, 23). This has been reproduced in a mouse in vitro processing system, and it has been demonstrated that, in this system, U3 is necessary for the ETS cleavage (17). RNase H cleavage of U3 in *Xenopus* oocytes (26) does not affect levels of mature rRNA but affects the processing pathway.

The function of U14 has been analyzed in some detail for *S. cerevisiae*. Depletion of U14 (19) gave a very similar phenotype to that of depletion of U3 (11). Essential elements of U14 have been identified by mutagenesis (14). They include the conserved boxes C and D, as well as domain A, which has strong complementarity to a region of 18S rRNA. Recently, Li and Fournier (18) showed that certain elements of mouse U14 could functionally replace elements of yeast U14 in a mouse-yeast hybrid U14.

Strains lacking snR10 are impaired in growth and in rRNA processing (30). A sextuple mutant, lacking snR3, snR4, snR5, snR8, snR9, and snR10, was not more impaired in growth than a strain lacking only snR10, and the role of the unessential snoRNAs is unknown (24).

Depletion of the nucleolar proteins NOP1 (the yeast homolog of fibrillarin) and GAR1 also resulted in a similar phenotype, that is, underaccumulation of 18S and its 20S precursor and elevated levels of an unusual pre-rRNA intermediate designated 23S (8, 32). Both of these proteins are associated with snoRNAs in the nucleolus (NOP1 with all known snoRNAs and GAR1 with a subset of snoRNAs). Expression of human or *Xenopus* fibrillarin can functionally replace NOP1 in *S. cerevisiae* (13). This, and the results for U14, demonstrates the functional conservation of snoRNPs and provides strong evidence that the basic mechanism of pre-rRNA processing has been conserved between *S. cerevisiae* and mammals.

It has been difficult to define the molecular events taking place during rRNA processing since so few essential snoRNP components have been characterized and depletion of any of these components results in a similar phenotype. An essential small nuclear RNA (snRNA), snR30, has previously been cloned from *S. cerevisiae* (1) but was not further characterized. No homologs in other organisms have been identified. This study was aimed at determining whether snR30 was a nucleolar species involved in rRNA

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processing and, if so, whether depletion of snR30 would result in a phenotype similar to that seen on depletion of the other essential snoRNP components.

MATERIALS AND METHODS

Strains and media. Standard *S. cerevisiae* growth and handling techniques were employed. Transformation was by the spheroplast method of Hinnen et al. (10). The haploid *SNR30*$^+$ strain carries *MAT***a** *ura3-52 leu2-3,112 ade1-100 his4-519* and is *GAL*$^+$ (strain BWG1-7A; kindly provided by L. Guarente). The strain used for snR30 depletion is isogenic except that it has the *URA3-pGAL10*::*snr30* construct integrated at the *SNR30* locus. Strain BWG1-7A is auxotrophic for uracil; therefore, an alternative strain was used for pulse-chase labeling of pre-rRNA with [^{3}H]uracil. This strain carries *MAT*α *leu2-3,112 his3-11,15 CAN1* (strain GRF18; kindly provided by R. Serrano).

For snR30 depletion, cells growing exponentially in galactose minimal medium were harvested by centrifugation, washed, and resuspended in glucose minimal medium. During growth, cells were diluted with prewarmed medium to maintain them in early exponential phase. The growth curve was drawn with the Cricket Graph program.

The D80 strain, which carries *MAT***a** *ura3-52 leu2-3,112 gal4-Δ537 gal7-Δ2*, was used for the cross-linking experiments since spheroplasts are more readily obtained from this strain than from the BWG1-7A strain.

The *GAL*::*nop1*, *GAL*::*gar1*, and *snr10* mutant strains used were those which had been previously constructed (8, 30, 32), and growth conditions were as described by those authors.

Cell fractionation. Cell fractionation and subnuclear fractionation were carried out as described by Hurt et al. (12) and Schimmang et al. (27).

Immunoprecipitation. Immunoprecipitation of snoRNAs with anti-NOP1 serum EC1.1 was performed as described by Schimmang et al. (27), with 3 μl of serum coupled to 15 μl of a packed volume of protein A-Sepharose (Sigma) and cell lysate equivalent to 0.5 optical density (at 600 nm) (OD_{600}) units of cells (10^7 cells). Lysate was prepared from strain BWG1-7A following growth in glucose minimal medium. Precipitated RNAs were recovered and analyzed as previously described (27).

In vivo cross-linking. In vivo cross-linking was carried out as previously described (2) with 4′-aminomethyl-4,5′,8-trimethyl-psoralen (HRI Associates Inc.). Spheroplasts, prepared from exponentially growing yeast cells, were treated with psoralen and either irradiated with UV light or held on ice in the dark for 15 min. The RNA was extracted and loaded on linear 10 to 30% sucrose gradients. Fractions were taken, and the RNA was precipitated and run on 1.2% agarose-formaldehyde gels. The RNA was transferred to Hybond-N (Amersham) filters for Northern (RNA) hybridization.

Construction of a *GAL*::*snr30* strain. Polymerase chain reaction was used to clone the region of the *SNR30* gene from position −294 to position +123. A *Bam*HI site at −32 and a *Hin*dIII site at −51 were introduced. Nucleotides −25 to −1 are deleted, resulting in a construct (pBS::30) in pBluescript KS(+) (Stratagene) which has the *Bam*HI site immediately 5′ to position +1 of *SNR30*. The *Hin*dIII-*Bam*HI fragment of pLGSD5, which contains the *URA3* gene and the *GAL10* promoter region (9), was isolated and cloned into the *Hin*dIII and *Bam*HI sites of pBS::30. This plasmid was digested with *Eag*I and *Xho*I, which released the linear fragment *SNR30* 5′ flanking sequence–*URA3-pGAL10-SNR30* 5′ coding sequence, and was used to transform yeast strain BWG1-7A, with selection for uracil prototrophy on galactose minimal medium.

Primer extensions. The primer extension to determine the 5′ end of snR30 was performed in a molar excess of primer as described by Boorstein and Craig (3) except that actinomycin D was omitted from the extension buffer. Annealing was carried out at 50°C, and extension was carried out at 46°C. After extension with avian myeloblastosis virus reverse transcriptase, the RNA was hydrolyzed and the DNA was precipitated and run on a sequencing gel. The same oligonucleotide was used to obtain the sequence ladder and was first phosphorylated with unlabeled ATP so as to ensure identical migration. The oligonucleotide used (GAAGCGC CATCTAGATG) is complementary to nucleotides 169 to 185 of snR30 (1).

Northern hybridization. RNA extraction, polyacrylamide and agarose-formaldehyde gel electrophoresis, and hybridization were performed as described by Tollervey (30). The vectors described by Tollervey et al. (32) were used to synthesize riboprobes for U3, U14, snR10, snR190, and U4 hybridizations. Polymerase chain reaction was used to clone the coding sequence of snR30, precisely flanked by *Eco*RI and *Hin*dIII restriction sites, into the riboprobe vector pT3/T7α-18 (Bethesda Research Laboratories, Inc.). This vector was then used to generate riboprobes for hybridization to snR30. The sequences of the oligonucleotides used as hybridization probes to the pre-rRNA are as follows: Oligo B, TCGGGTCTCTCTGCTGC; Oligo C, GCTCTTTGCTCT TGCC; Oligo D, ATGAAAACTCCACAGTG; Oligo E, CC AGTTACGAAAATTCTTG; and Oligo F, GGCCAGCAA TTTCAAGTTA.

Metabolic labeling of RNA. Cultures growing exponentially in galactose minimal medium were harvested, washed, and resuspended in prewarmed glucose minimal medium. The cells were subsequently maintained in an early exponential phase by dilution with prewarmed medium. Metabolic labeling with [*methyl*-^{3}H]methionine or [^{3}H]uracil was carried out 6 and 12 h after transfer to glucose medium as previously described (32), except that 4 ml of cells (OD_{600} ~ 0.3) was labeled with 125 μCi of label and chase times were 1, 2.5, 5.5, and 10 min.

RESULTS

snR30 is nucleolar and is associated with pre-rRNA. Subnuclear fractionation of yeast nuclei was performed to determine the subnuclear localization of snR30 (Fig. 1). Nuclei were purified by sedimentation through sorbitol and banding on sucrose step gradients (12, 27). As previously reported (1), snR30 is retained in the purified nuclei (Fig. 1, lane 2). To further fractionate nuclear components, the purified nuclei were extracted with Triton X-114, which releases nucleoplasmic, but not nucleolar, components (27). Triton X-114 extraction effectively released the nucleoplasmic snRNA U5 but not the nucleolar snoRNAs U3 and snR10 or snR30 (Fig. 1, lane 3). Extraction with the ionic detergent sodium deoxycholate is required for effective release of nucleolar components (27) and resulted in the release of the snoRNAs U3 and snR10 as well as snR30 (Fig. 1, lane 4). This indicates that snR30 is a nucleolar RNA.

The nucleolar protein NOP1 is associated with all known yeast snoRNAs but is not associated with the nucleoplasmic spliceosomal snRNAs (27). Immunoprecipitation with anti-NOP1 antibodies coprecipitated snR30 (Fig. 2). The

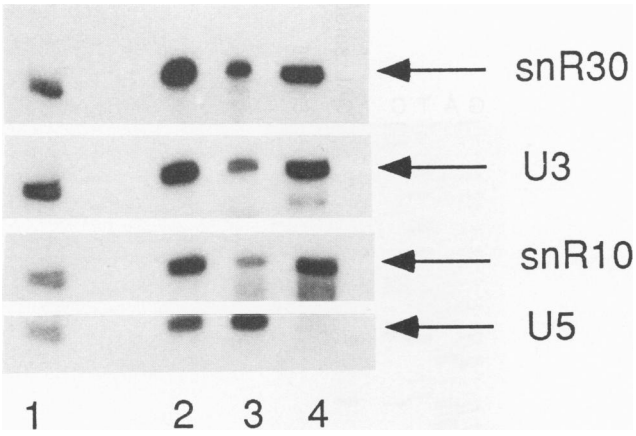


FIG. 1. Subnuclear fractionation of *S. cerevisiae*. Lane 1, total RNA recovered from whole cells; lane 2, RNA recovered from nuclei; lane 3, RNA recovered after Triton X-114 extraction of nuclei; lane 4, RNA recovered after sodium deoxycholate extraction of nuclei. Subnuclear fractionation was performed as described previously (12, 24), RNA was recovered and separated by polyacrylamide gel electrophoresis (PAGE). The RNA was transferred to a Hybond-N filter and hybridized with riboprobes to the indicated RNA species. Lanes 2 to 4 represent the same proportion of the starting preparation.

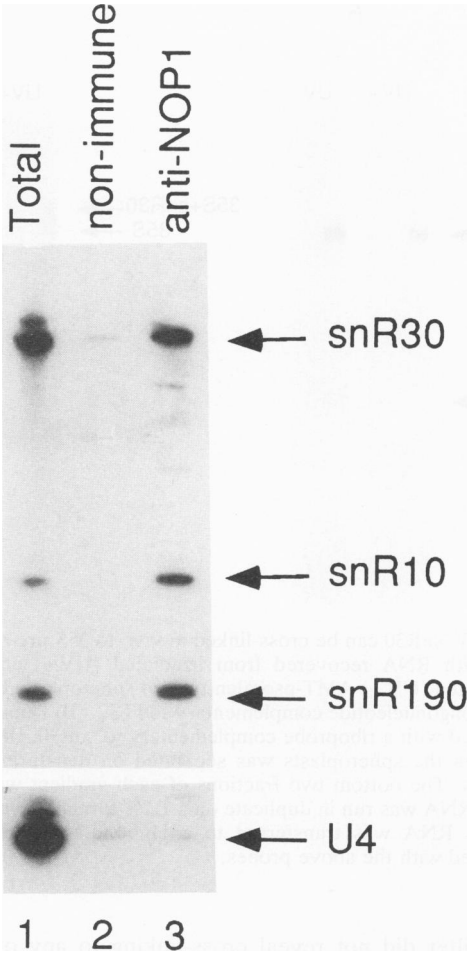


FIG. 2. Coprecipitation of snR30 with anti-NOP1 antibodies. Lane 1, total RNA extracted from cell lysate; lane 2, RNA recovered following immunoprecipitation with nonimmune serum; lane 3, RNA recovered following immunoprecipitation with anti-NOP1 antibodies. Following immunoprecipitations from a whole-cell lysate with anti-NOP1 antibodies, coprecipitated RNA was recovered, separated by PAGE, transferred to Hybond-N, and hybridized with antisense riboprobes for the indicated snoRNAs and snRNAs.

snoRNAs snR10 and snR190 were also coprecipitated by anti-NOP1 antibodies, but the nucleoplasmic snRNA U4 was not, demonstrating the specificity of the antibodies for nucleolar species. This is in agreement with the subnuclear fractionation data (Fig. 1), establishing snR30 as a member of the nucleolar class of snRNAs.

To determine whether snR30 was associated with pre-rRNA, cross-linking was carried out in vivo with aminomethyl trimethyl-psoralen (AMT-psoralen). AMT-psoralen has a planar ring structure and can intercalate between base-paired nucleic acids. When irradiated with UV light of 365 nm, the rings open and can interact with diagonally opposite pyrimidine residues to form a covalent cross-link. This results in the two base-paired species becoming covalently bound (4). In this experiment, the cross-linking was performed on intact yeast spheroplasts. As a control, AMT-psoralen was added to spheroplasts which were not irradiated. RNA was extracted from control and irradiated cells and run on denaturing sucrose gradients to separate the different pre-rRNA species. Fractions, containing different pre-rRNAs, were taken from both the control and the cross-linked gradients. RNA was recovered and analyzed by Northern hybridization with probes specific for pre-rRNA species or for snR30. For clarity, only the sucrose gradient fractions containing the 35S pre-rRNA are shown in Fig. 3. Filters were prepared in duplicate. One of the filters (Fig. 3A) was hybridized with an oligonucleotide complementary to ITS2 of the pre-rRNA (see oligonucleotide F in Fig. 8). It can be seen that the first of the two fractions contains the 35S pre-rRNA. The duplicate filter was hybridized with a riboprobe complementary to snR30 (Fig. 3B). This probe consistently cross-hybridized nonspecifically to 35S pre-rRNA and to 25S rRNA. In addition, however, it hybridized to an RNA species present in the cross-linked RNA (UV+) but not in the control (UV−), which migrated above the 35S pre-rRNA on the gel, at a position consistent with the addition of snR30 (606 nucleotides) to the ~7-kb 35S pre-rRNA. We estimate that approximately 1% of the 35S pre-rRNA and 0.1% of the snR30 are cross-linked. Similar low in vivo cross-linking efficiencies have been reported for U3 in rat, human, and yeast cells (2, 22, 29). For this reason, the cross-linked 35S species is not readily detected in Fig. 3A. The U3/35S cross-linked species migrates between the snR30/35S species and free 35S pre-rRNA (reference 2 and data not shown) and is also not detected in Fig. 3A. The low efficiency of cross-linking is also responsible for the poor ratio between the snR30 signal and the background hybridization to the 25S rRNA and 35S pre-rRNA. The upper band is reproducibly detected, but only with an snR30-specific probe and only in the UV-cross-linked sample. The possibility that this upper band is cross-linked mature 25S rRNA was ruled out by hybridizing the filter with an oligonucleotide probe specific for 25S rRNA. At an exposure which gives a signal for 25S rRNA equivalent to that of the snR30 probe (Fig. 3B), no band with the mobility of the cross-linked species can be detected (data not shown). We therefore believe this to be the result of snR30 becoming specifically cross-linked to the 35S pre-rRNA. Examination of the

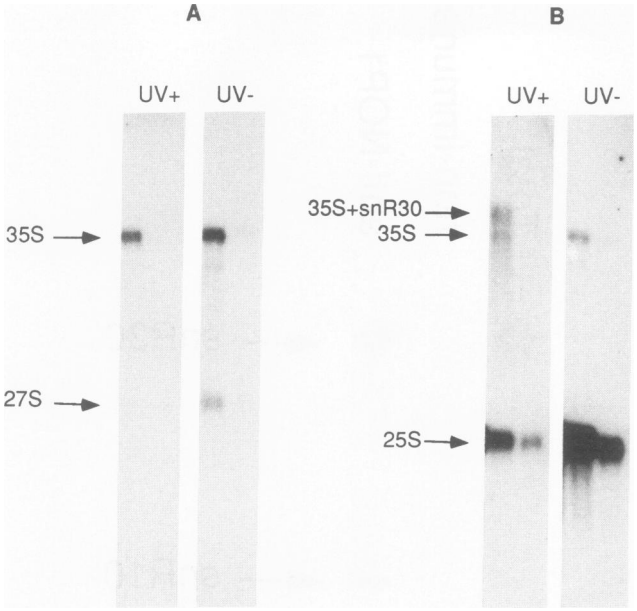


FIG. 3. snR30 can be cross-linked in vivo to 35S pre-rRNA. (A) Filter with RNA recovered from irradiated (UV+) and control unirradiated (UV−) AMT-psoralen-treated spheroplasts hybridized with an oligonucleotide complementary to ITS2. (B) Duplicate filter hybridized with a riboprobe complementary to snR30. RNA recovered from the spheroplasts was separated on denaturing sucrose gradients. The bottom two fractions of each gradient were taken, and the RNA was run in duplicate on a 1.2% formaldehyde-agarose gel. The RNA was transferred to a Hybond-N membrane and hybridized with the above probes.

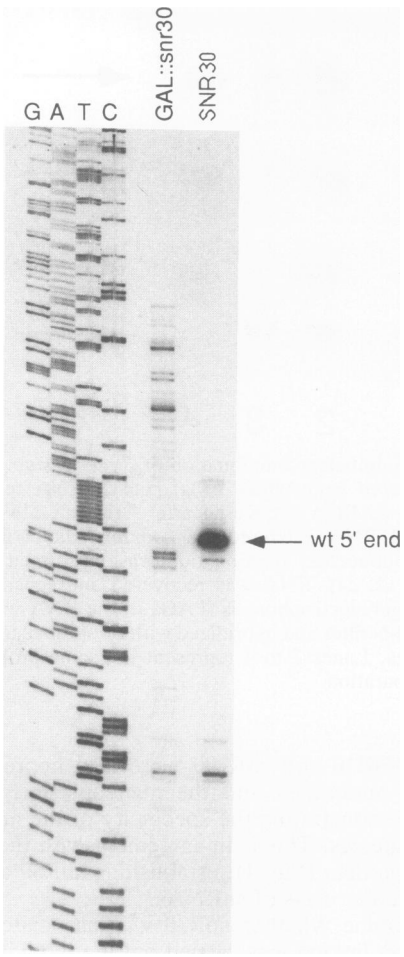


FIG. 4. Mapping the 5′ end of snR30 in the *GAL::snr30* strain. Primer extensions were performed on total RNA extracted from the wild type (*SNR30*) or from the *GAL::snr30* strain with an oligonucleotide complementary to nucleotides 169 to 185 of snR30. A plasmid containing *SNR30* was sequenced with the same oligonucleotide, and the sequence was used as a marker. The authentic 5′ end of snR30 is indicated.

entire filter did not reveal cross-linking to any other pre-rRNA species (data not shown).

Construction of a conditional *SNR30* allele. In order to determine whether snR30 plays a role in rRNA processing, it was necessary to construct a conditional allele which would allow snR30 to be specifically depleted from the cell. A construct was made in which the authentic *SNR30* promoter was replaced by the *GAL10* promoter. This promoter is active when the cells are grown on galactose but is repressed by growth on glucose. A fragment from the plasmid pLGSD5, containing the *URA3* gene, the *GAL10* promoter, and the *CYC* leader sequence, was inserted between the 5′ *SNR30* flanking region and the *SNR30* coding sequence. This was used to transform a haploid *ura3* strain of *S. cerevisiae*. *URA*$^+$ transformants were selected on galactose minimal medium. Transformants were confirmed to be galactose dependent, demonstrating that the construct had integrated at the *SNR30* locus, and one such strain was chosen for further study. The following experiments compared this strain (*GAL::snr30*) with the otherwise isogenic wild-type (*SNR30*) strain.

To determine the 5′ end of snR30 in the *GAL::snr30* strain, primer extensions were performed with equal quantities of total RNA extracted from the wild type (*SNR30*) or from the *GAL::snr30* strain (Fig. 4). As has previously been reported (7), transcription from this *GAL10* promoter construct does not initiate at a unique position, and as a result, there is a population of snR30 species with heterogeneous 5′ ends in the *GAL::snr30* strain. Only a small proportion of snR30 produced by the *GAL::snr30* strain has the authentic 5′ end. Comparison of the intensities of internal reverse transcriptase stops showed that the level of snR30 in the *GAL::snr30* strain is about 75% of the wild-type level. The level of active snR30 may be even lower since it is not known whether all of the heterogeneous snR30 species are functional. Despite this heterogeneity, and the lower overall level of snR30, the *GAL::snr30* strain grows at the same rate as the wild-type strain, both in complex and in minimal media, when galactose is the carbon source (data not shown).

The *GAL::snr30* strain was pregrown on galactose minimal medium and then transferred to glucose minimal medium to deplete snR30. For approximately two generations after transfer to glucose, the *GAL::snr30* strain grew identically to the wild-type strain, doubling every 2.5 h (Fig. 5). From 5 h on, there was a progressive increase in the doubling time of the *GAL::snr30* strain, whereas the wild type maintained a constant doubling time of 2.5 h. After 24 h of growth on glucose, the *GAL::snr30* strain had a doubling time of 12 h.

The levels of snR30, and other snoRNAs, during the period after transfer to glucose are shown in Fig. 6. Specific depletion of snR30 is observed. The level of snR30 drops

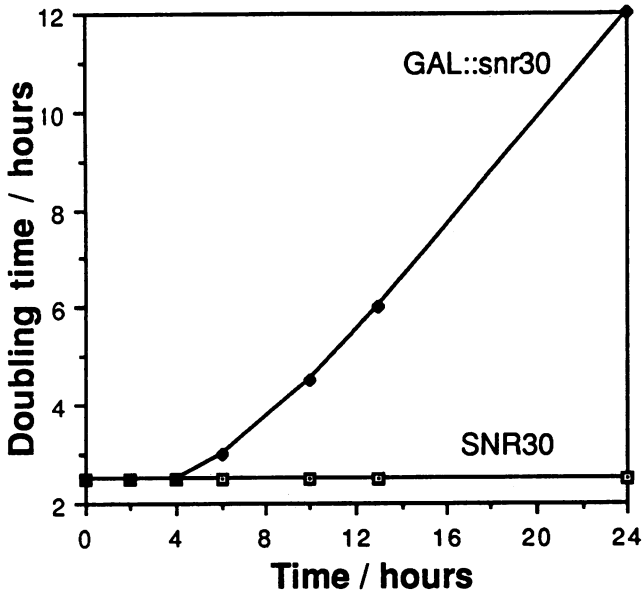


FIG. 5. Growth rate of the *GAL::snr30* strain on glucose medium. The growth rates of the *GAL::snr30* and of the wild-type (*SNR30*) strains following transfer from galactose to glucose minimal medium were monitored. Doubling times were calculated and plotted against time after transfer to glucose medium. The wild type continued to grow, with a doubling time of 2.5 h throughout the experiment.

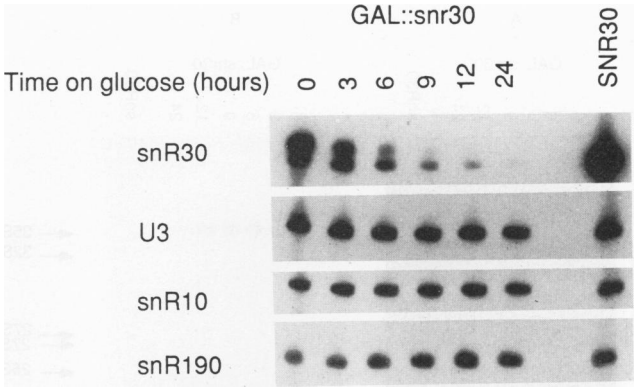


FIG. 6. Depletion of snR30 during growth on glucose medium. RNA was extracted from equal numbers of *GAL::snr30* cells (as judged by OD_{600} units) during the time course after transfer to glucose. The sampling times are indicated. The *GAL::snr30* strain growing on galactose is taken as 0 h. RNA was extracted from the wild-type (*SNR30*) strain following 12 h of growth on glucose. The RNA was separated by PAGE, transferred to a Hybond-N filter, and hybridized with riboprobes complementary to the snoRNAs indicated.

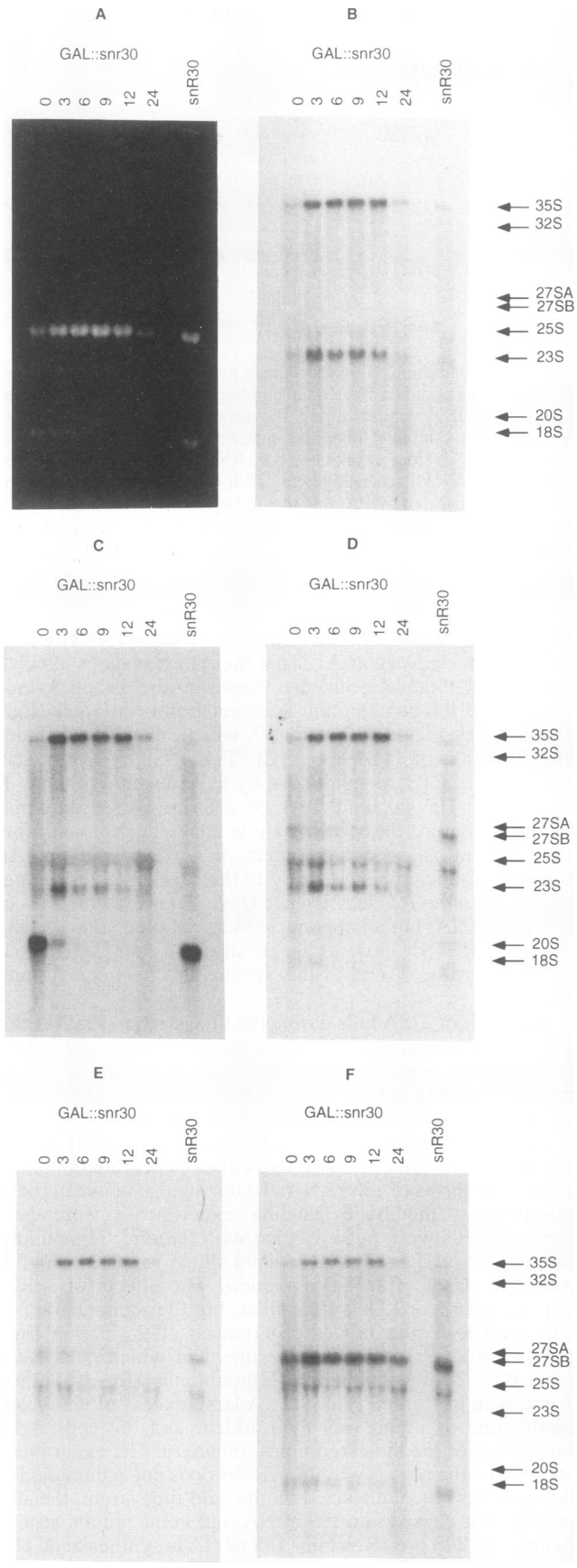
progressively, and after 6 h of growth on glucose, which is about the time when the growth rate starts to slow, snR30 is already at a low level. After 24 h of growth on glucose, snR30 is barely detectable. The levels of U3, snR10, and snR190 remain constant over this time period. The depletion of snR30 seems to occur more rapidly than would be expected if snR30 were merely being depleted by cell division; for example, between the 3- and 6-h time points, during which time the cells have doubled once, the level of snR30 falls by approximately 80%. This rapid depletion suggests that snR30 is relatively unstable in this strain, possibly because of the 5′ end heterogeneity.

rRNA processing in the *GAL::snr30* strain. The steady-state levels of mature rRNA and of pre-rRNA were analyzed during the time course of snR30 depletion (Fig. 7). The level of mature 25S rRNA rises immediately after shifting from galactose to glucose medium because of the effects of nutritional shiftup (21). Subsequently, the level of 25S rRNA remains constant during the time course of snR30 depletion, falling only after 24 h, by which time the growth rate of the cells is very low. The level of 18S, however, falls throughout snR30 depletion (Fig. 7A). The ratio of 25S to 18S, which is 1:1 when the cells are growing on galactose (0 h), rises significantly during snR30 depletion.

The same filter was hybridized successively with oligonucleotides complementary to different regions of the 35S pre-rRNA. The major processing pathway and the positions of the oligonucleotides are shown in Fig. 8. It is clear that there is an accumulation of the 35S species, showing that processing is slowed. There is no 32S pre-rRNA visible in the *GAL::snr30* strain, even at the zero time point (for example, the first lane in Fig. 7C), indicating that processing is already impaired when the cells are growing on galactose, though not to an extent that can slow growth. 32S pre-rRNA is formed by cleavage of 35S pre-rRNA at site A1; therefore, this impairment indicates that the *GAL::snr30* strain is inhibited in cleavage at A1. That the A1 cleavage is affected during snR30 depletion is also shown by the disappearance of 20S and the concomitant accumulation of a larger RNA species, designated 23S in Fig. 7, which, like 20S, includes the 5′ region of ITS1 (Fig. 7C). This 23S pre-rRNA also includes the ETS, as evidenced by hybridization to Oligo B (Fig. 7B). The accumulation of 23S pre-rRNA is rapid, occurring before the growth rate is affected, demonstrating that it is not a secondary effect of snR30 depletion. An oligonucleotide complementary to the region of ITS1 immediately 3′ to the A2 cleavage site (Fig. 7D) also hybridizes to 23S pre-rRNA, but an oligonucleotide complementary to the 3′ region of ITS1, close to the B1 site, does not (Fig. 7E). It follows that the 3′ end of this species lies between A2 and B1.

The level of 27SA falls during the time course (Fig. 7D and E), but there is no change in the level of 27SB (Fig. 7F). This is consistent with the previous data; cleavage at A2 is reduced, leading to lower levels of 27SA, but cleavage at B1, at the 5′ end of 5.8S, is unaffected in the mutant.

Accumulation of mature 18S rRNA is reduced in the mutant strain. This could be due either to degradation or to lack of synthesis of 18S rRNA. To distinguish between these possibilities, metabolic labeling experiments were performed. Cells were pulse-labeled with [*methyl*-^{3}H]methionine, or with [^{3}H]uracil, 6 h (data not shown) and 12 h (Fig. 9) after the onset of snR30 depletion. The effects of snR30 depletion for 6 or 12 h were similar, but the accumulation of 35S pre-rRNA was not as pronounced at the earlier time point. Figure 9 shows an experiment in which cells were labeled for 2 min with [*methyl*-^{3}H]methionine (Fig. 9A) or for 1 min with [^{3}H]uracil (Fig. 9B). A large excess of unlabeled methionine or uracil was then added, and the cells were incubated for the indicated times (minutes). The experiment shows that the reason why 18S rRNA does not accumulate is because it is not synthesized; in the wild-type strain, the 20S precursor is chased into 18S rRNA, but in the mutant strain, neither the 20S pre-rRNA nor 18S rRNA is synthesized. The accumulation of 35S pre-rRNA appears more pronounced



when the cells are labeled with uracil, but this merely reflects the fact that nascent transcripts which were labeled during the pulse continue to be extended after addition of cold uracil. The 23S pre-rRNA can be detected in labeling experiments, but only after longer exposures (data not shown). Since 23S pre-rRNA is visible on Northern blots at the same time points (e.g., Fig. 7C), this marked underaccumulation in metabolic labeling experiments indicates either that 23S pre-rRNA is synthesized slowly or that it is degraded rapidly. Because its rate of synthesis must correspond to that of 27SB pre-rRNA, and 27SB pre-rRNA is being synthesized at normal rates (Fig. 9), then it must be concluded that 23S pre-rRNA is rapidly degraded. The in vivo labeling data agree with the Northern data regarding synthesis of 25S rRNA. The 27SA pre-rRNA is not visible in the mutant strain, but 27SB pre-rRNA appears to be processed normally into mature 25S rRNA. Depletion of the nucleolar snoRNP protein NOP1 interferes with the methylation of pre-rRNA (32). Comparison of labeling with [*methyl*-^{3}H]methionine (Fig. 9A) and [^{3}H]uracil (Fig. 9B) indicates that cells depleted of snR30 are not impaired in the methylation of pre-rRNA.

Previous work has reported that an aberrant 23S pre-rRNA also accumulates in an *snr10* mutant strain and on depletion of the nucleolar ribonucleoprotein components NOP1, GAR1, and U3 (8, 11, 30, 32). It was proposed, but not demonstrated, that the 3′ end of this species was at B1 at the 3′ end of ITS1. To determine whether this was in fact the case, RNA was extracted from strains depleted of NOP1 or GAR1 and from a strain lacking snR10. The RNA was analyzed by Northern hybridization with oligonucleotide probes to regions of ITS1 (Fig. 10). As was the case with the 23S pre-rRNA seen on depletion of snR30, oligonucleotides complementary to the 5′ region of ITS1 (Fig. 10A) and to the region of ITS1 immediately 3′ to cleavage site A2 (Fig. 10B) hybridize to the 23S species but an oligonucleotide complementary to the 3′ region of ITS1 (Fig. 10C) does not. This result strongly suggests that the 23S pre-rRNA that accumulates on depletion of snR30 is the same as the 23S species that has been previously identified (8, 11, 30, 32). It was proposed that the 21S pre-rRNA species that accumulates in

FIG. 7. Steady-state levels of mature and precursor rRNA from wild-type (*SNR30*) and *GAL::snr30* strains during the time course of snR30 depletion. RNA was extracted from equal numbers of cells (as judged by OD_{600} units). (A) Mature RNA visualized by ethidium bromide staining. (B) Hybridization with an oligonucleotide complementary to the 5′ ETS (Oligo B). (C) Hybridization with an oligonucleotide complementary to the 5′ region of ITS1 (Oligo C). (D) Hybridization with an oligonucleotide complementary to the region of ITS1 immediately 3′ to the A2 cleavage site (Oligo D). (E) Hybridization with an oligonucleotide complementary to the 3′ region of ITS1 (Oligo E). (F) Hybridization with an oligonucleotide complementary to ITS2 (Oligo F). RNA was extracted from the wild type following 12 h of growth on glucose medium. RNA was extracted from the *GAL::snr30* strain 3, 6, 9, 12, and 24 h after transfer to glucose medium. RNA extracted from the *GAL::snr30* strain growing on galactose is taken as 0 h of depletion. RNA was extracted from equal numbers of cells, as judged by OD_{600} units. The RNA was separated on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N filter. The filter was hybridized in turn with oligonucleotides complementary to various regions of the pre-rRNA. The positions of the mature rRNA and the pre-rRNA are indicated in the figure. The positions of the oligonucleotides on the pre-rRNA are indicated in Fig. 8, and their sequences are given in the Materials and Methods section.

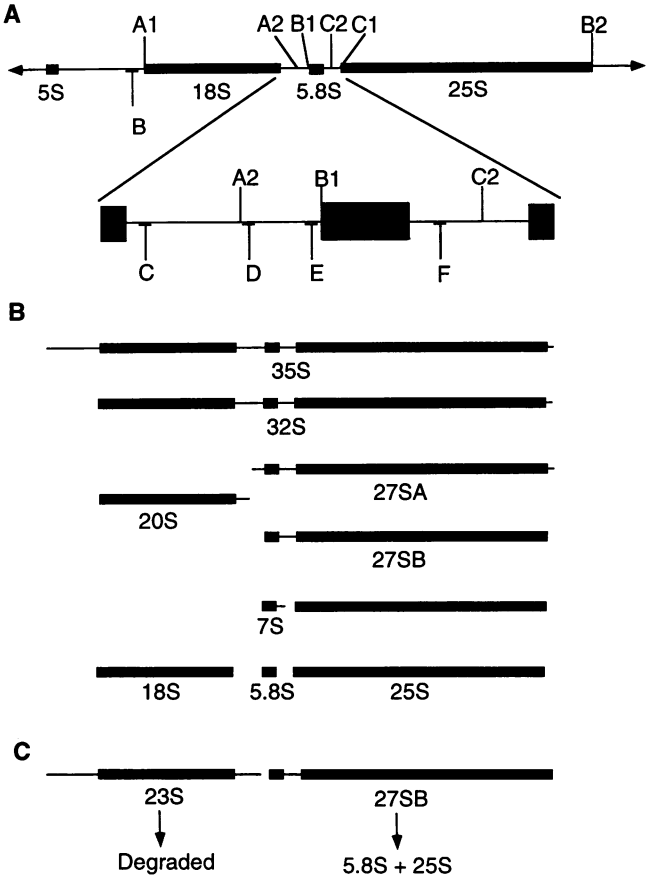


FIG. 8. The rRNA processing pathway in *S. cerevisiae*. (A) Organization of a single repeat of genes coding for rRNA. The 5S gene is transcribed by RNA polymerase III. The 18S, 5.8S, and 25S rRNAs are transcribed by RNA polymerase I as a single transcript (35S) containing a 5′ ETS, the 18S rRNA, the first internal transcribed spacer (ITS1), the 5.8S rRNA, the second internal transcribed spacer (ITS2), the 25S rRNA, and the 3′ ETS. The internal transcribed spacers are not labeled in the figure. The major characterized cleavage sites A1, A2, B1, B2, C1, and C2 are indicated. The positions of the oligonucleotides used as hybridization probes in this study are also indicated: B (5′ ETS); C, D, and E (ITS1); and F (ITS2). (B) The major processing pathway. The initial 35S transcript is cleaved at the A1 site to yield 32S, which is rapidly cleaved at A2, giving the 20S and 27SA intermediates. 20S is transported to the cytoplasm, where it undergoes maturation to 18S rRNA. 27SA is cleaved at sites B1 and B2, producing 27SB. Cleavage of 27SB at sites C1 and C2 generates the 7S intermediate and mature 25S rRNA. The 7S intermediate is processed to mature 5.8S rRNA. (C) The two cleavage products of 35S in the *GAL::snr30* strain. The 3′ end of 23S pre-rRNA has not been accurately determined. The 27SB pre-rRNA is processed normally into mature 5.8S and 25S rRNA, but the 23S pre-rRNA is degraded.

the *snr10* mutant strain ran from the A1 site to the B1 site (30), but, as is the case with 23S, Fig. 10 shows that the 3′ end of this species is between sites A2 and B1. It is likely that 21S has the same 3′ end as 23S.

DISCUSSION

snR30 is the 11th characterized member of the yeast snoRNA family. Subnuclear fractionation shows that it cofractionates with the nucleolar snoRNAs rather than the nucleoplasmic snRNAs, and snR30 can be coprecipitated by antibodies against the nucleolar proteins NOP1 (this study) and GAR1 (8). It is the fourth yeast snoRNA that has been shown to play a role in rRNA processing.

As is the case with all essential snoRNP components analyzed to date (U3, U14, snR10, NOP1, and GAR1), snR30 is implicated in the cleavage events that produce 18S rRNA. Depletion of any of these components results in underaccumulation of 20S pre-rRNA and of 18S rRNA and appearance of an aberrant 23S pre-rRNA intermediate. The 35S pre-rRNA is cleaved to give the 23S and 27SB pre-rRNAs; 23S pre-rRNA extends beyond cleavage site A2 into the 3′ region of ITS1, and the 5′ end of 27SB is at site B1, the 3′ end of ITS1. Cleavage of the B1 site is correct at the nucleotide level in strains depleted of snR30, snR10, NOP1 (our unpublished results), or GAR1 (8), and this led to the supposition that the 3′ end of 23S was at B1 (8, 11, 30, 32). This, however, is not the case, as demonstrated by the failure of oligonucleotide E to hybridize to 23S pre-rRNA in strains depleted of snR30 (Fig. 7E), NOP1, or GAR1 (Fig. 10C) or in a strain lacking snR10 (Fig. 10C). The 3′ end of 23S pre-rRNA must lie within ITS1, between the regions complementary to oligonucleotides D and E (Fig. 8A), and not at B1. The 3′ end of 23S pre-rRNA may be generated by an aberrant cleavage that takes place only when one of these snoRNP components is depleted or by a cleavage event in ITS1 that has hitherto gone undetected. It is also possible that, after cleavage of 35S at B1, an exonuclease rapidly degrades the 3′ region of ITS1, resulting in a 23S species whose 3′ end lies within ITS1. This question is currently being addressed.

A previous study (2) used psoralen cross-linking techniques to cross-link U3 to pre-rRNA. It was found that U3 can be cross-linked to the 35S pre-rRNA. The authors went on to map the cross-linking sites in detail. U3 is cross-linked to two sites within the ETS. That the cross-linking represents a functionally significant interaction is shown by the fact that, like depletion of U3 (11), mutation of one of these ETS sites (at +470) disrupts synthesis of 18S rRNA. The results presented here show that snR30 can also be cross-linked to the 35S pre-rRNA. The cross-linking sites have not yet been mapped, but it is worth noting that there is a region in the ETS (+480 to +486), immediately adjacent to the U3 binding site at +470 to +479, which shows sequence complementarity to the 5′ region of snR30 (+2 to +8). In a mouse cell-free system, processing of a site in the ETS has been shown to require the assembly of a large complex including U3 and fibrillarin (16). The similarities in the phenotypes of all six characterized yeast snoRNP components (the proteins NOP1 and GAR1 and snoRNAs U3, U14, snR10, and snR30) suggest the possibility that these too may form a large processing complex. Such a complex might assemble on the ETS and be responsible for the cleavages that generate 18S rRNA.

It is remarkable that none of the snoRNP components that have previously been analyzed has been shown to be involved in the cleavage events that produce the 5.8S or 25S rRNA. This is also the case for the strain depleted of snR30; 27SB pre-rRNA is processed normally into 25S rRNA (Fig. 7 and 9), and 7S pre-rRNA is processed normally into 5.8S rRNA (data not shown). It is possible that all the components thus far analyzed are associated in one processing complex and that another, as yet undetected, complex carries out the B and C cleavages. There are a number of proteins which have been shown to be necessary for cleavages other than those at A1 and A2: the *RRP2* gene product appears to be

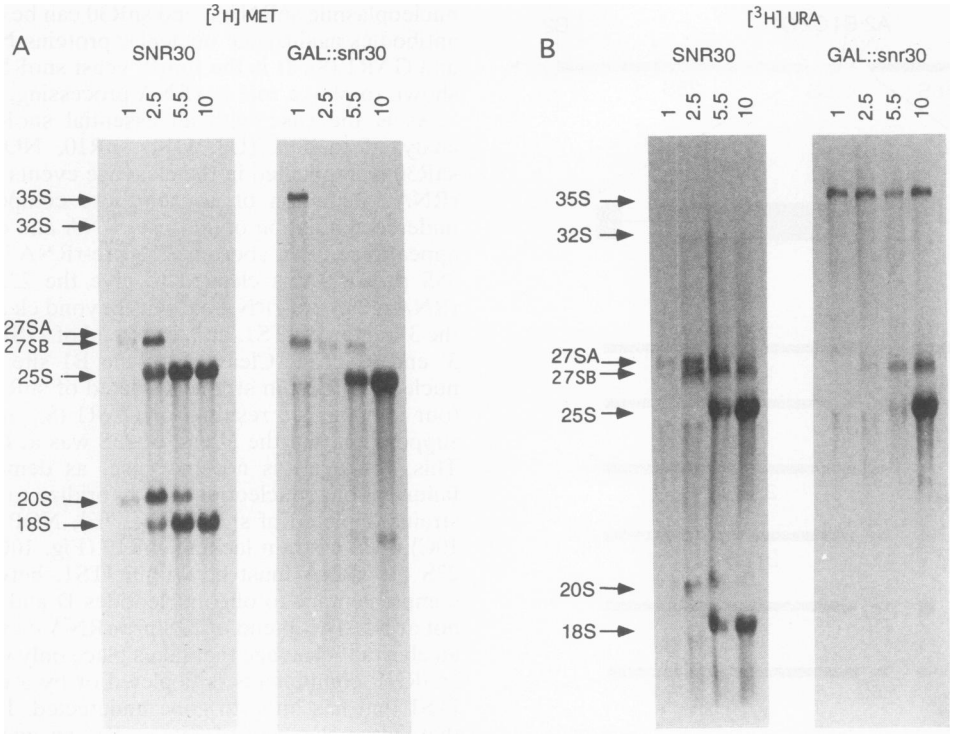


FIG. 9. Metabolic labeling of pre-rRNA from wild-type (*SNR30*) and *GAL::snr30* strains during the time course ot depletion. Labeling was performed on the *SNR30* strain and *GAL::snr30* strain 12 h after transfer to glucose medium. Cells were pulse-labeled with [*methyl*-^{3}H]methionine for 2 min (A) or with [^{3}H]uracil for 1 min (B) at 30°C. A large excess of unlabeled methionine or uracil was then added, and the cells were incubated for 1, 2.5, 5.5, or 10 min as indicated. RNA was extracted, run on a 1.2% agarose-formaldehyde gel, and transferred to a GeneScreen Plus filter. RNA was visualized by fluorography. The positions of the major precursors and of the mature rRNA are indicated.

involved in cleavage of B1 at the 5′ end of 5.8S (20, 28), and the products of two genes, *RRP1* (6) and *SPB4* (25), are necessary for synthesis of 25S rRNA but not of 18S rRNA. A temperature-sensitive allele of *RRP1* can be suppressed by a mutation in the *SRD1* gene (5). Until antibodies against these proteins become available, it will not be possible to determine whether they are snoRNP components or whether they are processing enzymes which act independently of snoRNPs.

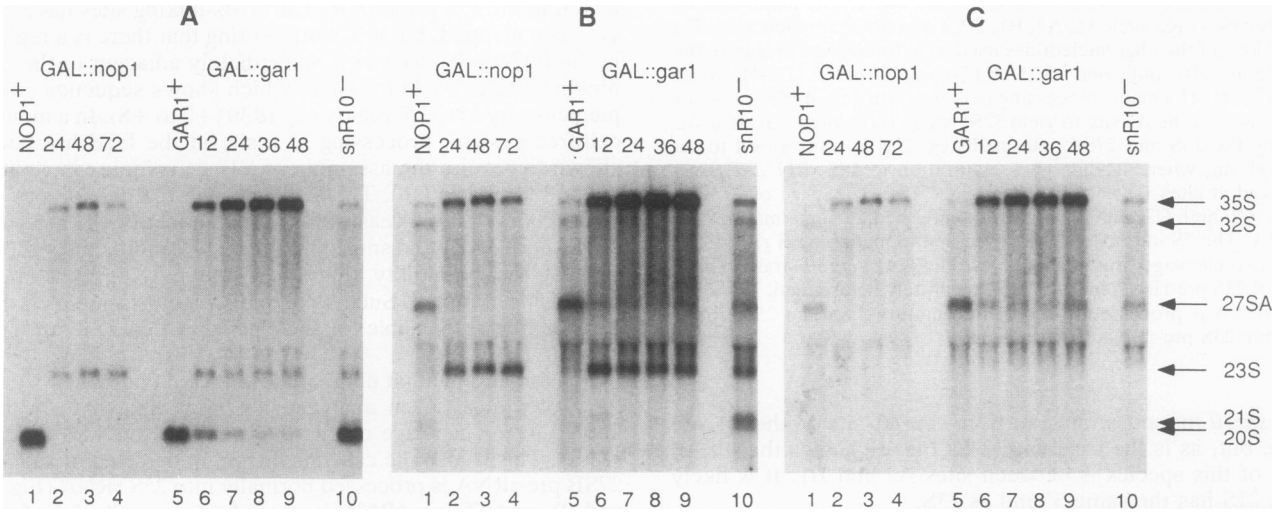


FIG. 10. Steady-state levels of precursor rRNA in NOP1- and GAR1-depleted strains and in an *snr10* mutant strain. (A) Filter with RNA extracted during the time course of depletion hybridized with an oligonucleotide complementary to the 5′ region of ITS1 (Oligo C). (B) The same filter hybridized with an oligonucleotide complementary to the region of ITS1 immediately 3′ to the A2 cleavage site (Oligo D). (C) The same filter hybridized with an oligonucleotide complementary to the 3′ region of ITS1 (Oligo E). RNA was extracted from strains depleted of the nucleolar protein NOP1 (lanes 1 to 4) or GAR1 (lanes 5 to 9) at the indicated times after onset of protein depletion or from a strain that lacks snR10. The RNA was separated on a 1.2% agarose-formaldehyde gel and transferred to a Hybond-N filter. The filter was hybridized successively with oligonucleotides complementary to different regions of ITS1. The positions of the oligonucleotides are indicated in Fig. 8.

Although U3, U14, snR10, NOP1, GAR1, and snR30 are all involved in synthesis of 18S rRNA, it may be misleading to assume that the phenotypes of depletion are identical at the molecular level. It is possible that there are subtle differences between the phenotypes, and work to analyze the cleavage events and processing intermediates in strains depleted of snoRNP components in more detail is in progress. This work should contribute to the eventual elucidation of the mechanisms of rRNA processing not only in *S. cerevisiae* but, given the conservation of components, also in higher eukaryotes.

ACKNOWLEDGMENTS

We thank Thomas Dandekar for assistance in making the *GAL::snr30* construct; Hanna Lehtonen for expert technical assistance; Iain Mattaj, Heather Wood, and Yves Henry for critical reading of the manuscript; John Hughes for provision of an *SNR30* clone; and Susan Weston and Samantha O'Loughlin for synthesis of oligonucleotides.

REFERENCES

1. **Bally, M., J. Hughes, and G. Cesareni.** 1988. snR30: a new essential small nuclear RNA from Saccharomyces cerevisiae. Nucleic Acids Res. **16:**5291–5303.
2. **Beltrame, M., and D. Tollervey.** 1992. Identification and functional analysis of two U3 binding sites on yeast pre-ribosomal RNA. EMBO J. **11:**1531–1542.
3. **Boorstein, W. R., and E. A. Craig.** 1989. Primer extension analysis of RNA. Methods Enzymol. **180:**347–369.
4. **Cimino, G. D., H. B. Gamper, S. T. Isaacs, and J. E. Hearst.** 1985. Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. Annu. Rev. Biochem. **54:**1151–1193.
5. **Fabian, G. R., S. M. Hess, and A. K. Hopper.** 1990. srd1, a Saccharomyces cerevisiae suppressor of the temperature-sensitive pre-rRNA processing defect of rrp1-1. Genetics **124:**497–504.
6. **Fabian, G. R., and A. K. Hopper.** 1987. *RRP1*, a *Saccharomyces cerevisiae* gene affecting rRNA processing and production of mature ribosomal subunits. J. Bacteriol. **169:**1571–1578.
7. **Faye, G., D. Leung, K. Tatchell, B. Hall, and M. Smith.** 1981. Deletion mapping of sequences essential for in vivo transcription of the iso-1-cytochrome c gene. Proc. Natl. Acad. Sci. USA **78:**2258–2262.
8. **Girard, J. P., H. Lehtonen, M. Caizergues-Ferrer, F. Amalric, D. Tollervey, and B. Lapeyre.** 1992. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. EMBO J. **11:**673–682.
9. **Guarente, L., R. R. Yocum, and P. Gifford.** 1982. A GAL10-CYC1 hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site. Proc. Natl. Acad. Sci. USA **79:**7410–7414.
10. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA **75:**1929–1933.
11. **Hughes, J. M. X., and M. Ares, Jr.** 1991. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5′ external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. EMBO J. **10:**4231–4239.
12. **Hurt, E. C., A. McDowall, and T. Schimmang.** 1988. Nucleolar and nuclear envelope proteins of the yeast Saccharomyces cerevisiae. Eur. J. Cell Biol. **46:**554–563.
13. **Jansen, R. P., E. C. Hurt, H. Kern, H. Lehtonen, M. Carmo-Fonseca, B. Lapeyre, and D. Tollervey.** 1991. Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast. J. Cell Biol. **113:**715–729.
14. **Jarmolowski, A., J. Zagorski, H. V. Li, and M. J. Fournier.** 1990. Identification of essential elements in U14 RNA of Saccharomyces cerevisiae. EMBO J. **9:**4503–4509.
15. **Kass, S., N. Craig, and B. Sollner-Webb.** 1987. Primary processing of mammalian rRNA involves two adjacent cleavages and is not species specific. Mol. Cell. Biol. **7:**2891–2898.
16. **Kass, S., and B. Sollner-Webb.** 1990. The first pre-rRNA-processing event occurs in a large complex: analysis by gel retardation, sedimentation, and UV cross-linking. Mol. Cell. Biol. **10:**4920–4931.
17. **Kass, S., K. Tyc, J. A. Steitz, and B. Sollner-Webb.** 1990. The U3 small nuclear ribonucleoprotein functions at the first step of preribosomal RNA processing. Cell **60:**897–908.
18. **Li, H. V., and M. J. Fournier.** 1992. U14 function in Saccharomyces cerevisiae can be provided by large deletion variants of yeast U14 and hybrid mouse-yeast U14 RNAs. EMBO J. **11:**683–689.
19. **Li, H. V., J. Zagorski, and M. J. Fournier.** 1990. Depletion of U14 small nuclear RNA (snR128) disrupts production of 18S rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**1145–1152.
20. **Lindahl, L., R. H. Archer, and J. M. Zengal.** 1992. A new rRNA processing mutant of Saccharomyces cerevisiae. Nucleic Acids Res. **20:**295–301.
21. **Ludwig, J. R., II, S. G. Oliver, and C. S. McLaughlin.** 1977. The regulation of RNA synthesis in yeast II: amino acids shift up experiments. Mol. Gen. Genet. **158:**117–122.
22. **Maser, R. L., and J. P. Calvet.** 1989. U3 small nuclear RNA can be psoralen cross-linked *in vivo* to the 5′ external transcribed spacer of pre-ribosomal RNA. Proc. Natl. Acad. Sci. USA **86:**6523–6527.
23. **Miller, K. G., and B. Sollner-Webb.** 1981. Transcription of mouse rRNA genes by RNA polymerase I: in vitro and in vivo initiation and processing sites. Cell **27:**165–174.
24. **Parker, R., T. Simmons, E. O. Shuster, P. G. Siliciano, and C. Guthrie.** 1988. Genetic analysis of small nuclear RNAs in *Saccharomyces cerevisiae*: viable sextuple mutant. Mol. Cell. Biol. **8:**3150–3159.
25. **Sachs, A. B., and R. W. Davis.** 1990. Translation initiation and ribosomal biogenesis: involvement of a putative rRNA helicase and RPL46. Science **247:**1077–1079.
26. **Savino, R., and S. A. Gerbi.** 1990. In vivo disruption of Xenopus U3 snRNA affects ribosomal RNA processing. EMBO J. **9:**2299–2308.
27. **Schimmang, T., D. Tollervey, H. Kern, R. Frank, and E. C. Hurt.** 1989. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. EMBO J. **8:**4015–4024.
28. **Shuai, K., and J. W. Warner.** 1991. A temperature sensitive mutant of S. cerevisiae defective in pre-rRNA processing. Nucleic Acids Res. **19:**5059–5064.
29. **Stroke, I. L., and A. M. Weiner.** 1989. The 5′ end of U3 snRNA can be crosslinked *in vivo* to the external transcribed spacer of rat ribosomal RNA precursors. J. Mol. Biol. **210:**497–512.
30. **Tollervey, D.** 1987. A yeast small nuclear RNA is required for normal processing of pre-ribosomal RNA. EMBO J. **6:**4169–4175.
31. **Tollervey, D., and E. C. Hurt.** 1990. The role of small nucleolar ribonucleoproteins in ribosome synthesis. Mol. Biol. Rep. **14:** 103–106.
32. **Tollervey, D., H. Lehtonen, M. Carmo-Fonseca, and E. C. Hurt.** 1991. The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. EMBO J. **10:**573–583.