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Fragments of the Internal Transcribed Spacer 1 of Pre-rRNA Accumulate in *Saccharomyces cerevisiae* Lacking 5′→3′ Exoribonuclease 1

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The portion of the internal transcribed spacer 1 found on 20S pre-rRNA accumulates in *Saccharomyces cerevisiae* lacking 5′→3′ exoribonuclease 1, showing that an endonucleolytic cleavage at the 3′ terminus of 18S rRNA is involved in the 20S pre-rRNA to 18S mature rRNA conversion. Smaller fragments of the spacer sequence are also found. The exoribonuclease may be involved as a cytoplasmic RNase in the hydrolysis of the spacer.

The suggested metabolic roles (see reference 13) of a 5′→3′ exoribonuclease are crucial to cells, but no studies clarifying the function of this basic biochemical reaction are available. As a step toward determining the metabolic role of a 160-kDa yeast protein with 5′→3′ exoribonuclease activity (18, 19), a *Saccharomyces cerevisiae* gene, *XRN1*, was first cloned and then disrupted to yield a haploid yeast strain lacking the enzyme (13); this strain was designated *xrn1*. Although the gene is not essential, its absence markedly affected the cell growth rate. Sequencing of the *XRN1* gene (12) has shown its identity with the *DST2* and *SEP1* genes, cloned and disrupted by Dykstra et al. (6) and Tishkoff et al. (20), respectively, which encode a protein with DNA strand exchange activity (5, 11). The findings of these authors suggest that the protein is involved in genetic recombination. The cloning and sequencing by Kim et al. (9) of the *KEM1* gene that affects nuclear fusion in *S. cerevisiae* have shown that it is also the same gene. That diverse effects are caused by the loss of the gene suggests that the protein is multifunctional, and Johnson and Kolodner (8) find that it also has 5′→3′ deoxyribonuclease activity. RNase activity was not measured in the other laboratories. Since it has high-level RNase activity, we have continued investigations of the effect of loss of the gene on RNA metabolism in *xrn1* strains and report here the results of studies of pre-rRNA processing.

The purification by Lasater and Eichler (14) of a 5′→3′ exoribonuclease from nucleoli of mouse Ehrlich ascites tumor cells suggested a role for such an enzyme in pre-rRNA processing, possibly involving 5′-trimming reactions to remove all or part of the external and internal transcribed spacers (ETS and ITSs, respectively; see pre-rRNA structure in Fig. 2A) and/or hydrolysis of fragments of the ETS and ITSs generated by endoribonucleolytic cleavages. The rapid hydrolysis of the spacers of the pre-rRNAs following cleavage in vivo has made a study of the exact location of the cleavage sites for generation of the 5′ and 3′ termini of the mature rRNA molecules very difficult. Studies by Bowman et al. (1) and Raziuddin et al. (15) suggest that the 5′ and 3′ termini of 18S rRNA in mouse L cells are formed from endonucleolytic cleavages at the exact ends of the mature species. However, by using sequential incubations of an 18S rRNA precursor fragment with nucleolar and cytoplasmic extracts of HeLa cells, Hannon et al. (7) found that the 5′ terminus of human 18S rRNA is formed in vitro by an initial cleavage (nucleolar) close to the 5′ end followed by a 5′→3′-trimming reaction (cytoplasmic). In yeast, 20S pre-rRNA containing 209 nt of the ITS1 at the 3′ end has been detected as the precursor of the 18S mature rRNA (3, 22). No studies are available showing whether the 18S rRNA is formed by an exact endonucleolytic cleavage of the 20S pre-rRNA removing the 209-nt fragment or whether a 3′-trimming reaction may be involved.

By using the *xrn1* yeast strain lacking the exoribonuclease, pre-rRNA processing has been analyzed to try to detect 5′-trimming errors or accumulation of spacer sequences. We find an accumulation of fragments of ITS1, the largest one being 209 to 210 nt, showing that an endonucleolytic cleavage at the 3′ terminus of the 18S rRNA can convert the 20S pre-rRNA to the 18S mature rRNA in *S. cerevisiae*.

Accumulation of ITS1 fragments of the 20S pre-rRNA. In an attempt to detect faulty processing at the 5′ termini of 5.8S rRNA in *xrn1* cells, poly(A)$^-$ RNAs of wild-type *S. cerevisiae* [YNN27 (α *ura3-52 trp1-289*)] and the *xrn1* strain (*YNN27 xrn1 ΔBgl::URA3*) were labeled with [^{3}H]adenine and examined by gel electrophoresis with 6% polyacrylamide gels (Fig. 1A). ^{3}H-labeled RNA bands (as determined by sensitivity to RNase A) slightly above the 5.8S rRNA were found in the *xrn1* poly(A)$^-$ RNA. The total ^{3}H label in the accumulated bands was about 80 to 90% of that of the 5.8S rRNA label. Gel analysis by ethidium bromide staining was also used to detect the accumulation of the small RNA species. Results found with RNAs isolated from cultures of both wild-type *S. cerevisiae* and the *xrn1* strain at 23, 30, and 36°C are shown in Fig. 1B. Four RNA bands accumulate at both 23 and 30°C (lanes 4 and 5). At 36°C (lane 6), the two larger bands are dominant. Since the bands were about 200 nt in length and accumulated in large amounts, it seemed most likely that they were 5.8S pre-rRNA fragments or fragments derived from the 209-nt sequence of the ITS1 found on the 20S pre-rRNA. The RNA bands were labeled at about the same rate as the 5.8S and 5S rRNAs (Figs. 1A and 1B) of the *xrn1* strain, which has a restricted growth rate (13).

Five small oligodeoxynucleotide probes (oligoprobes) (20 to 22 nt long) complementary to sequences of the ETS and ITSs were synthesized and used for Northern (RNA) analysis of the accumulated RNAs. The oligoprobes are shown in Fig. 2A (top); the probes are complementary to two portions

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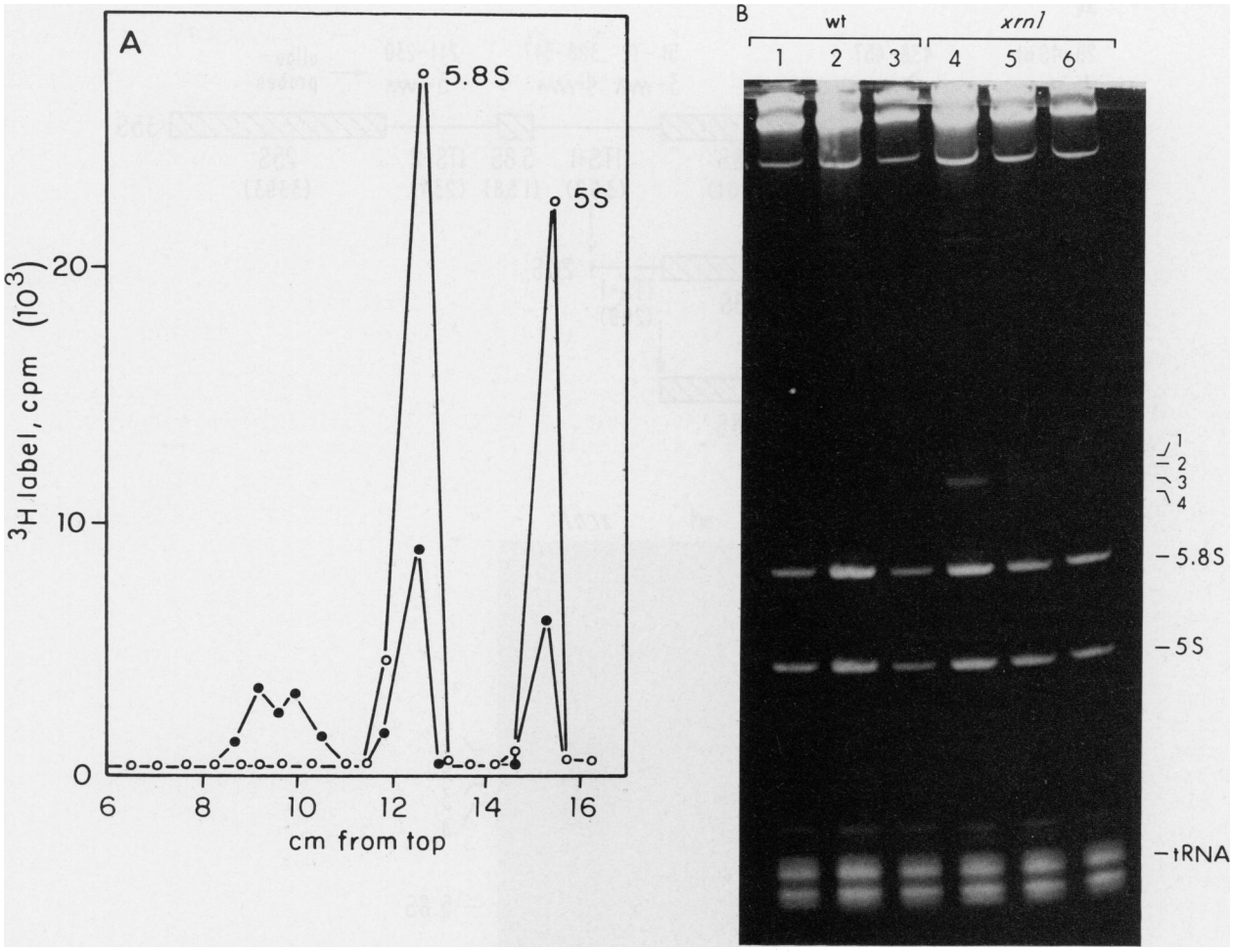

FIG. 1. Accumulation of small RNA species in *xrn1* cells. (A) [^{3}H]adenine labeling and analysis of small RNA species. Wild-type (wt) and *xrn1* cells were grown in YPD medium (17) to 1.3×10^7 cells/ml at 30°C. To 10-ml portions of each culture, 100 μg of adenine and 200 μCi of [2,8-^{3}H]adenine (ICN, 221 Ci/nmol) were added, and the cultures were allowed to grow for 2 h. At that time, the cells were collected by centrifugation and suspended in 0.5 ml of 50 mM sodium acetate–10 mM EDTA–1% sodium dodecyl sulfate. RNA was extracted and subjected to oligo(dT)-cellulose chromatography as described by Domdey et al. (4). The poly(A)$^-$ RNA fractions (20 μg) were electrophoresed by using a 6% polyacrylamide-urea gel (16). The portion of the gel containing the small RNA bands was sliced, and the radioactivity was eluted by heating the gel slices for 30 min at 80°C in 0.5 ml of 1 N NaOH. After neutralization, radioactivity was determined. ○, wt; ●, *xrn1*. (B) Small RNA bands accumulated by *xrn1* cells at 23, 30, and 36°C. Cultures (20 ml) of wt and *xrn1* cells were grown to 1.5×10^7 cells/ml, and RNA was extracted as described above. Samples (20 to 25 μg) were analyzed by gel electrophoresis as described above, and ethidium bromide (5 μg/ml) was used to stain the gel. Lanes 1 and 4, 23°C; lanes 2 and 5, 30°C; lanes 3 and 6, 36°C.

of the ETS, two portions of ITS1, and one portion of ITS2 (see the legend to Fig. 2 for exact locations). Also shown is the yeast rRNA-processing scheme involving 18S rRNA formation from the 35S pre-rRNA. Northern blots of RNA samples from wild-type and *xrn1* cells grown at 30°C showed that none of the oligoprobes except probe 3 (complementary to the part of ITS1 found on the 20S pre-RNA) had significant reactivity. Figure 2B (*xrn1* lane) shows the reactivity of probe 3 with the accumulated RNA bands of *xrn1* depicted in Fig. 1. All four of the predominant bands (bands 1 through 4 from the top) reacted with the probe, the reactivity being about the same as the amount detected from ethidium bromide staining. No reactivity was found with wild-type RNA (Fig. 2B, wt lane). The results show that *xrn1* cells accumulate fragments of the ITS1, and the sizes of the fragments (~200 nt) suggested that they were derived from the hydrolysis of the 20S pre-rRNA to the 18S mature rRNA. DeJonge et al. (3) and Veldman et al. (22) showed that the ITS1 sequence of the 20S pre-rRNA is 209 nt in length.

The sizes of the RNA fragments accumulated by the *xrn1* cells were determined by labeling the RNA with $^{32}P_i$ and examining it by using an 8% sequencing gel (16). The results are shown in Fig. 3A (lane 1; lane 2 is a shorter exposure). A sequencing reaction of spinach phosphoribulose kinase cDNA was run as a size reference and is shown at the left; the 150- and 200-nt bands are marked. The largest accumulated RNA band sizes were at 209 and 210 nt, appearing as a doublet, and the other main bands were at about 206, 198, and 191 to 193 nt (lane 1). The doublet bands may be due to one band having a terminal phosphate (5.8S RNA appears at 159 nt with the terminal phosphate) or to two bands with a 1-nt difference. The shorter fragments of the spacer most likely result from limited hydrolysis of the accumulated 209- and 210-nt fragment at the 3′ and/or 5′ ends, although alterna-

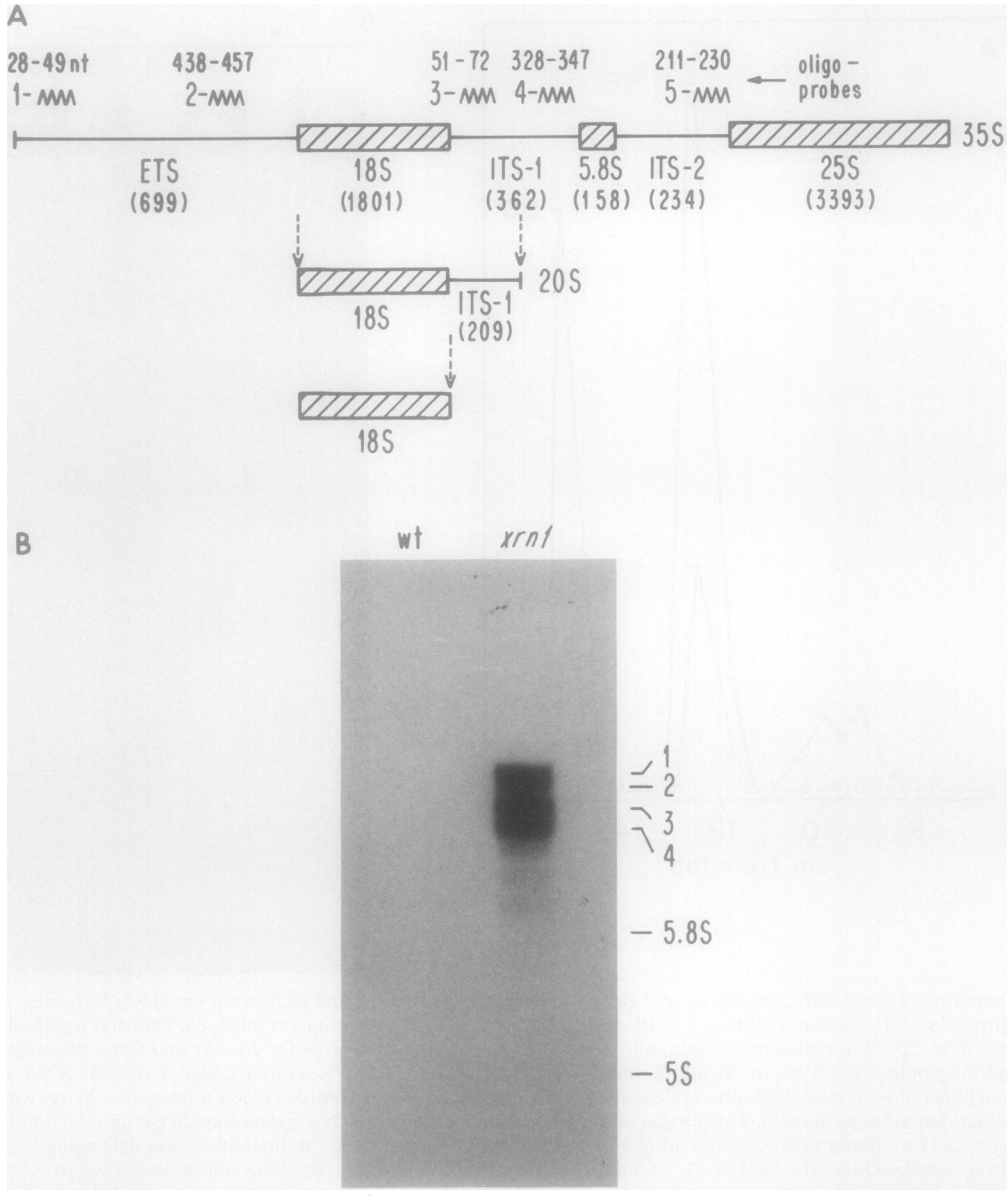

FIG. 2. Detection of ITS1 sequences as the accumulated RNA species in *xrn1* cells. (A) Diagram showing the pre-rRNA processing scheme as adapted from Klootwijk and Planta (10) and the location of complementary oligodeoxynucleotide probes (synthesized in an Applied Biosystems 391 DNA synthesizer) used for analysis of the accumulated bands. At the top, the complementary nucleotides contained in the oligoprobes are given as the location in nucleotides from the 5′ end of the ETS and ITSs. Zigzag lines depict the approximate locations of the probes with respect to the spacer sequences just below in the 35S pre-rRNA. For example, probe 1 is complementary to nucleotides 28 to 49 from the 5′ end of the ETS. Probe 3 is complementary to nucleotides 51 to 72 from the 5′ end of the ITS1 or from the 18S rRNA-ITS1 junction. All the numbers shown are in nucleotides, with the values below the spacers and rRNAs of the 35S pre-rRNA being the lengths in nucleotides of each. (B) Northern blot of accumulated RNA bands using probe 3. Wild-type (wt) and *xrn1* RNAs (30°C growth) were electrophoresed as described in Fig. 1. The RNA bands on the gel were transferred to a Zeta-Probe membrane by using an LKB Novablot electrophoresis transfer unit. The membrane was hybridized with 5′-^{32}P-labeled probe 3 as described by Church and Gilbert (2) and subjected to autoradiography.

tive endonucleolytic cleavage sites in the 20S pre-rRNA cannot be excluded. Primer extension analysis of the four accumulated RNA bands was carried out using probe 3 as a primer to determine whether the smaller fragments (RNA bands 2 through 4 [Fig. 3A]) were shorter at the 5′ ends. Since probe 3 is complementary to nucleotides 51 to 72 from the 5′ end of the ITS1 or the 18S rRNA-ITS1 junction, a primer extension product of 72 nt would be expected if the 5′ end of the RNA fragments were coincident with the 5′ end of ITS1. The primer extension of RNA bands 1 and 2 (Fig. 3B, lanes 2 and 3) yielded a fragment of approximately 70 to 72 nt. The fragment with band 1 was detected on a longer exposure (lane 2b). The low reactivity of band 1 may have been due to a poor recovery on its elution from the preparative gel. The shorter extension products found with band 1 RNA, possibly resulting from RNA degradation or from pausing or prema-

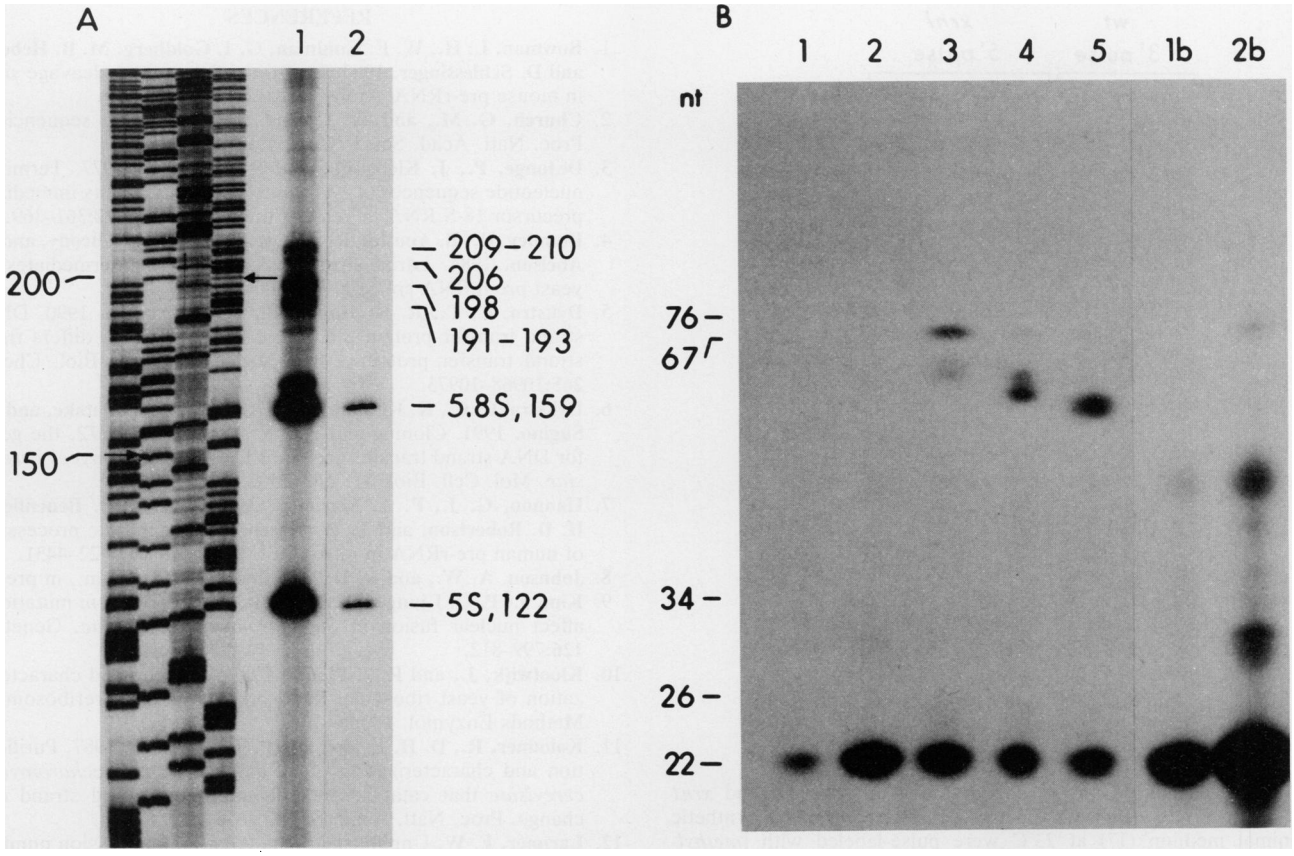

FIG. 3. Gel analysis of the sizes of the accumulated RNA bands and primer extension analysis of 5′-ends. (A) Sequencing gel analysis of the sizes of the accumulated RNA species. The *xrn1* cells were grown in low-phosphate YPD medium (17) to 3.5×10^6 cells/ml. To 40 ml of culture, 1 mCi of $^{32}P_i$ (ICN) was added, and the cells were grown to 1.2×10^7 cells/ml. The cells were then collected, and poly(A)$^-$ RNA was prepared by phenol extraction and oligo(dT)-cellulose chromatography as described in Fig. 1. The RNA (3×10^5 cpm) was then examined on an 8% sequencing gel (lane 1; lane 2 is a shorter exposure). (B) Primer extension analysis of the 5′ termini of the accumulated RNA species. 5′-^{32}P-labeled probe 3 (1.2 pmol), complementary to nucleotides 51 to 72 from the 5′ terminus of ITS1, was hybridized with 0.5 to 2 pmol (as determined from ethidium bromide staining of the gel) of RNA bands 1 through 4 (isolated from a gel similar to that shown in Fig. 1B), and the hybridized samples were subjected to primer extension (16). The samples were electrophoresed on a 15% polyacrylamide–urea gel using 5′-^{32}P-labeled pBR322 (*Msp*I-cut) markers. The locations and sizes (in nucleotides) of the marker bands are shown at the left. The gel was subjected to autoradiography. Lane 1, ^{32}P-labeled primer; lanes 2 through 5, primer extension with bands 1 through 4, respectively; lanes 1b and 2b, longer exposures of lanes 1 and 2.

ture termination by reverse transcriptase, were found with all of the RNA bands on the longer exposure. The results suggest that both RNA bands 1 and 2 have 5′ termini the same as that of ITS1. Band 2, from its size, must then be 3 or 4 nt shorter at the 3′ terminus. The primer extension of RNA bands 3 and 4 (Fig. 3B, lanes 4 and 5) yielded fragments that are shorter by 8 to 10 and 11 to 15 nt, respectively, at the 5′ termini. It is possible that these two RNA bands are also slightly shorter at the 3′ termini to account for their sizes (Fig. 3A). The results show that the cytoplasmic conversion of the 20S pre-rRNA to the 18S rRNA species (21) can involve an endonucleolytic cleavage of the entire fragment and that the fragment accumulates in the *xrn1* strain lacking 5′→3′ exoribonuclease-1. No accumulation of spacer sequences has been described previously.

Rates of pre-rRNA-processing reactions. To determine the relative rates of the different pre-rRNA-processing reactions and to determine whether the same rRNA precursors were found in the *xrn1* cells as in wild-type cells, pulse-chase labeling of cells with [*methyl*-^{3}H]-methionine followed by gel electrophoresis of the ^{3}H-labeled RNA was carried out as described by Warner (23). Wild-type and *xrn1* yeast cultures were labeled at 23°C for 3 and 5 min, respectively. Unlabeled methionine was then added, and samples were taken at the times shown for RNA analysis. The results (Fig. 4) show that the same labeled RNA species are found in the *xrn1* strain as in the wild-type strain. The rate of 18S rRNA formation is slowed slightly relative to that of 25S rRNA formation. From densitometric scans of the gel shown, the percentages that the 25S and 18S rRNA species were of the total 27S + 25S and 20S + 18S rRNAs, respectively, were calculated for wild-type RNA at 3 min and *xrn1* RNA at 5 min. These values were as follows: wt 25S, 72%; *xrn1* 25S, 84%; wt 18S, 79%; and *xrn1* 18S, 66%. Relative to the rate of 25S rRNA formation, the 20S pre-rRNA-to-18S rRNA conversion is slowed by 25 to 30%. Similar results were obtained in two additional experiments.

As described above, a role for a 5′→3′ exoribonuclease in 5′-trimming reactions during rRNA precursor processing was suggested by Lasater and Eichler (14) and, more recently, by Hannon et al. (7), who found a cytoplasmic activity that removed several nucleotides of the ETS1 from

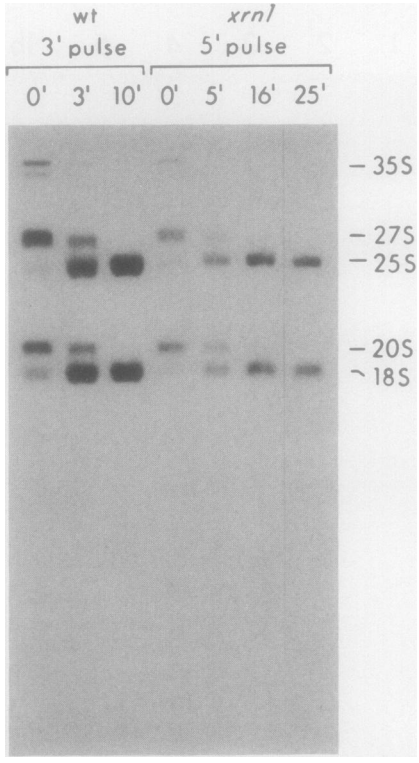

FIG. 4. Pre-rRNA processing in the wild-type (wt) and *xrn1* strains of *S. cerevisiae*. wt and *xrn1* cells (10^7 cells/ml) in synthetic minimal medium (17) at 23°C were pulse-labeled with [*methyl*-^{3}H]methionine as described by Warner (23). The pulse-label (60 μCi/ml) was for 3 min (wt) or 5 min (*xrn1*) before the addition of cold methionine (500 μg/ml). At the times shown above the lanes (in minutes), 1 ml of each culture was cooled rapidly and collected. RNA was extracted as described by Domdey et al., (4), and the RNA species were fractionated on a formaldehyde–agarose gel (1.5% agarose). The gel was soaked in En^{3}Hance (NEN-DuPont), dried, and exposed to X-ray film at −80°C.

a mouse cell pre-rRNA species. To determine whether the 18S and 25S mature rRNAs of the *xrn1* cells had the correct 5′ termini, primer extension analysis of these two RNA fractions was done. The experiment showed that >95% of the 5′ termini of the two rRNA species are correct in the *xrn1* cells (data not shown). The gel analysis in Fig. 3A shows that the size of the 5.8S rRNA is correct.

The results show that an exact endonucleolytic event is involved in the conversion of the 20S pre-rRNA to 18S mature rRNA, since the 209-nt portion of the ITS1 found on the 20S pre-rRNA accumulates in *S. cerevisiae* lacking 5′→3′ exoribonuclease 1. These studies suggest that the exoribonuclease is involved in the hydrolysis of the cleaved spacer or that its absence affects the level of another protein involved in this hydrolysis.

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