Sequence and Generation of Mature Ribosomal RNA Transcripts in Dictyostelium discoideum

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The amoeba Dictyostelium discoideum is a well established model organism for studying numerous aspects of cellular and developmental functions. Its ribosomal RNA (rRNA) is encoded in an extrachromosomal palindrome that exists in ~100 copies in the cell. In this study, we have set out to investigate the sequence of the expressed rRNA. For this, we have ligated the rRNA ends and performed RT-PCR on these circular RNAs. Sequencing revealed that the mature 26 S, 17 S, 5.8 S, and 5 S rRNAs have sizes of 3741, 1871, 162, and 112 nucleotides, respectively. Unlike the published data, all mature rRNAs of the same type uniformly display the same start and end nucleotides in the analyzed AX2 strain. We show the existence of a short lived primary transcript covering the rRNA transcription unit of 17 S, 5.8 S, and 26 S rRNA. Northern blots and RT-PCR reveal that from this primary transcript two precursor molecules of the 17 S and two precursors of the 26 S rRNA are generated. We have also determined the sequences of these precursor molecules, and based on these data, we propose a model for the maturation of the rRNAs in Dictyostelium discoideum that we compare with the processing of the rRNA transcription unit of Saccharomyces cerevisiae.

Ribosomes belong to the most important molecular machines in the cell. As the sites of protein biosynthesis, they are highly conserved, and the analysis of their RNA constituents allows the determination of phylogenetic relationships over large evolutionary distances. Within the last decade, beautiful crystallographic work has confirmed that the catalytic activity resides with the RNA and that the ribosome thus is a ribozyme at heart (1–3). Early indications for this came from the observation that the peptidyltransferase reaction was surprisingly resistant to treatment with proteinase and phenol, procedures that are expected to degrade proteins (4).

To produce the amounts of ribosomal RNAs that are required to ensure constant protein biosynthesis is very challenging for each organism. For most metazoan, efficient rRNA transcription is facilitated by clustering multiple rDNA copies within their chromosomes. In principle, this solution is also implemented for the amoeba Dictyostelium discoideum (5, 6); here, however, the rDNA clusters are localized on extrachromosomal elements, rather than within the chromosomes. These show a size of 88 kb with a palindromic organization of two rRNA transcription units on the mirror-symmetric arms (7, 8). Each nucleus contains ~100 copies of the palindrome, resulting in about 200 copies of the rRNA genes per cell. In addition to these, a chromosomal copy of the rDNA exists in Dictyostelium (9) that likely is the source of the extrachromosomal elements. Extrachromosomal rDNA is also found in other amoebe like Physarum (10) or the ciliate Tetrahymena (11, 12), whereas Entamoeba features circular rDNA templates (13).

In higher eukaryotes, the genes for the 5 S rRNA and the other rRNAs are frequently unlinked (Ref. 14 and references therein), although they are still connected in the palindrome of Dictyostelium (5), similar to the situation in yeast (15). Despite this physical association, there are two transcription start sites, also in the amoeba (14). As is the case for all eukarya studied so far, the 5 S rRNA is transcribed by RNA polymerase III, although the remaining rRNAs (17 S, 5.8 S, and 26 S) are generated as a 7.5 kb large 37 S primary transcript by RNA polymerase I (16). The 37 S RNA is thought to encompass the externally transcribed spacers (ETS)3 at the 5’ end before the 17 S rRNA and at the 3’ end after the 26 S rRNA, respectively (Fig. 1). Thus, also included are the two internally transcribed spacers, ITSI and ITSII, which reside between the 17 S and 5.8 S rRNA sequences, or the 5.8 S and the 26 S rRNA sequences, respectively.

The processes that generate the mature rRNA from such a primary transcript have been studied in great detail in the yeast Saccharomyces cerevisiae, where a consecutive series of endo- and exonucleolytic cleavage reactions leads to the removal of spacer regions surrounding the rRNAs (17, 18). Next to these, a considerable number of rRNA residues are modified by the action of H/ACA box or C/D box small nucleolar ribonucleoprotein that leads to site-specific pseudo-uridylation and 2’-O-ribose methylation (17–19). Many small nucleolar RNAs and their verified or predicted targets have also been identified in D. discoideum (20).

The relative orientation of the cistrons within the RNA polymerase I-transcribed rRNA transcription unit is similar.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–7, Table 1, and an additional reference.
between S. cerevisiae and D. discoideum, although their genomic localization varies, being chromosomal and extrachromosomal, respectively. Not only is the overall organization of the extrachromosomal rDNA palindrome in Dictyostelium known (8) but its detailed sequence has also been analyzed by Kuspa and co-workers (7). Earlier work has shown that the processing of the 37 S primary transcript proceeds via distinct precursors, overall in a manner similar to that of yeast (16, 21). Unlike for yeast, neither the sequences of the intermediates nor those of the mature rRNA of the amoeba are described unambiguously in the literature. Because annotated sequences also do not coincide with the migration behavior of isolated RNA from the amoeba, we have set out here to investigate the rRNAs of D. discoideum. We have determined the sequences of all rRNAs in the AX2 strain of the amoeba and identified several precursor molecules. Based on these data, we propose a scheme for the processing of the primary transcript to yield mature rRNA.

**EXPERIMENTAL PROCEDURES**

**Growth**—The D. discoideum strain AX2 was grown axenically in HL5 medium containing 50 μg/ml ampicillin, 250 ng/ml amphotericin, 10 μg/ml penicillin, and 10 μg/ml streptomycin at 22 °C in shaking suspension.

**Oligonucleotides**—DNA oligonucleotides were purchased from Invitrogen and are listed in [supplemental Table S1](#).

**Isolation of Genomic DNA**—Genomic DNA was isolated as described previously (22).

**Isolation of Total RNA**—In all cases, RNA was isolated from exponentially growing cultures with a density of 2 × 10⁶ cells/ml. For total RNA, 2 × 10⁷ cells were harvested and washed in ice-cold phosphate buffer (2 mM Na₂HPO₄, 15 mM KH₂PO₄, (pH 6.7)), and the cell pellet was resuspended in 1 ml of TRIzol reagent (Invitrogen). RNA was isolated according to the manufacturer’s instructions, and the concentration of the isolated RNA was determined spectrophotometrically. RNA was separated by denaturing agarose gel electrophoresis (5 V/cm) in the absence of ethidium bromide to rule out an interference of the dye with the migration of the RNA. After complete separation, the samples were visualized by staining with ethidium bromide.

**Isolation of Nuclear RNA**—To isolate nuclei, we followed a protocol developed by C. Pears.⁴ In brief, 6 × 10⁷ cells were harvested and washed in ice-cold phosphate buffer and lysed in 2 ml of nuclear isolation buffer (50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 3 mM MgCl₂, 3 mM CaCl₂, 0.5 M sorbitol, 0.6% Triton X-100, protease inhibitor (Roche Applied Science)). Nuclei were centrifuged at 2300 × g for 5 min and resuspended in 1 ml of TRIzol reagent (Invitrogen). Subsequently, RNA was treated as described before.

**Isolation of Nuclear Depleted RNA**—To generate an RNA fraction that is largely depleted of nuclei, 5 × 10⁸ cells were harvested, washed in ice-cold phosphate buffer, and lysed in 10 ml of an ice-cold solution containing 50 mM HEPEs (pH 7.5), 40 mM MgCl₂, 20 mM KCl, 5% saccharose, and 1% Nonidet P-40. Nuclei were immediately separated by centrifugation at 5900 × g for 20 min, and the supernatant was used to isolate RNA by TRIzol reagent as described above.

**DNase I Digestion**—Total RNA (60 μg) was incubated with 3 units of RNase-free DNase I (Fermentas) in 70 μl of 10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 100 μM CaCl₂ for 45 min at 23 °C. After extractions with phenol and chloroform, RNA was precipitated with ethanol and recovered in 60 μl of ultra-pure water.

**RNase-H Cleavage**—To cut RNA in DNA-RNA hybrids, 3 μg of total RNA were mixed with 350 ng of a given DNA oligonucleotide in 20 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, incubated for 5 min at 75 °C, and then annealed for 5 min at 37 °C. The mixture was then treated for 30 min at 37 °C with 200 units of Moloney MLV RT, which exhibits RNase H activity (Invitrogen). After extraction with phenol and chloroform, RNA was precipitated with ethanol. The products were separated by electrophoresis in an agarose gel and visualized after staining with ethidium bromide.

**cRT-PCR**—To generate RNA ends suitable for circularization, 15 μg of DNase I-treated total RNA was dephosphorylated by incubation with 1 unit of SAP (Fermentas) in 30 μl of 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 μg/ml BSA for 30 min at 37 °C. After precipitation, the RNA was incubated with 20 units of T4 polynucleotide kinase (Fermentas) and 20 units of Riboprobe RNase Inhibitor (Fermentas) in 30 μl of 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT, 100 μM spermidine, 1 mM ATP for 30 min at 37 °C. After protein extraction with phenol and chloroform, RNA was precipitated with ethanol. 2 μg of RNA were incubated with 20 units of T4 RNA ligase (Fermentas) and 20 units of Riboprobe RNase inhibitor in 20 μl of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 20 mM ATP, 100 μg/ml BSA for 30 min at 37 °C. After extraction with phenol and chloroform, RNA was precipitated with ethanol. Reverse transcription and PCR amplification were performed as described below (cDNA synthesis) with the following cycling conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 50 °C for 1 min, and final step with 72 °C for 5 min. The PCR products were ligated in the pGEM-T Easy vector (Promega) and transformed in chemically competent Escherichia coli TOP10 cells (Invitrogen). Plasmid DNA was isolated with the GeneJET plasmid miniprep kit (Fermentas) according to the manufacturer’s instructions and then digested with EcoRI (Fermentas) to detect positive clones. Subsequently, positive clones were sequenced by Seqlab (Göttingen, Germany) using the DNA oligonucleotide p33 ([supplemental Table S1](#)).

**cDNA Synthesis**—For cDNA synthesis, 2 μg of DNase I-treated total RNA and 2 μl of a complementary oligonucleotide (5 pmol/μl) were incubated in 25 μl using 200 units Moloney MLV reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Generation of cDNA from the 17 S precursor in dependence of a 3’-terminal polyadenylation was performed by using complementary primers with (p38) or without a poly(T)₁₅ part (p37) in addition to seven sequence-specific nucleotides. A temperature of 42 °C was used for cDNA synthesis. The subsequently performed PCRs (25 μl) contained 1 μl of cDNA product, 12.5 pmol of forward/reverse primer, 250 μM dNTPs, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P-40, and 1 μl of TaqDNA

⁴ C. Pears, personal communication.
polymerase. PCR was performed with the following cycling conditions: 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final step with 72 °C for 5 min. Products were separated by electrophoresis in an agarose gel and visualized after staining with ethidium bromide.

Northern Analysis—Northern blot analysis was performed as described previously (23). As probes, DNA oligonucleotides were 5’ end-labeled by incubating 10 pmol of primer with 10 units of T4 polynucleotide kinase (Fermentas) in 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 5 mM DTT, 100 μM spermidine, and 0.37 MBq of [γ-32P]ATP. The complete reaction volume was placed on a Sephadex G-50 column (Fluka) and centrifuged at 1000 rpm for 3 min. After prehybridization of the membrane with Church buffer containing 250 mM (Na+ and H+) phosphate buffer (pH 7.0), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) SDS for at least 1 h at 42 °C, the purified oligonucleotide probe was added and incubated at 42 °C overnight. The membrane was washed twice with 2× SSC in 0.1% SDS for 15 min each, twice with 1× SSC in 0.1% SDS for 10 min, and finally twice with 0.5× SSC in 0.1% SDS for 5 min. After that, it was exposed to a PhosphorImager screen (Fujifilm).

RNA Sequencing—Full-length small RNAs have been isolated from the D. discoideum strain AX4, using exponentially growing amoeba with a density of 1–3 × 10⁶ cells/ml. The RNAs were 3’-C-tailed, and an RNA oligonucleotide was ligated to their 5’ ends before being converted to cDNA, cloned, and sequenced (20).

Primer Extensions—For primer extension, 4 μg of either total or nucleus-enriched RNAs were used as template. RNA was denatured for 1 min at 95 °C in 50 mM Tris-HCl (pH 8.6), 60 mM NaCl, and 10 mM DTT (10-μl reaction volume) in the presence of radiolabeled first strand primer that was gel-purified before use. Annealing was performed at 57 °C for 4 min. To this, 5 μl of 5 mM dNTP (each), 5 μl of 25 mM MgCl2, and 5 μl of AMV-RT (1 unit/μl) in 50 mM Tris-HCl (pH 8.6), 60 mM NaCl, and 10 mM DTT were added. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by denaturation at 80 °C for 10 min. Upon RNase A treatment at 37 °C for 15 min, reaction products were recovered by ethanol precipitation. To generate an RNA sequencing ladder, this protocol was followed to the annealing step. To this mixture, 5 μl of 1.67 mm dNTP (each), 5 μl of 1 mm of individual ddNTP, and 5 μl of Maxima RT (1 unit/μl; Fermentas) in 50 mM Tris-HCl (pH 8.6), 60 mM NaCl, 5 mM MgCl2, and 10 mM DTT were added. The reaction mixture for each nucleotide was further treated as described above.

Reaction products were separated by denaturing gel electrophoresis using polyacrylamide gels at concentrations of 5, 10, or 20%.

RESULTS

Mapping Oligonucleotide Probes to the Predicted Large rRNAs from D. discoideum—When total RNA from the D. discoideum wild type strain AX2 is separated in a denaturing agarose gel, the 17 S and 26 S rRNAs migrate at heights of ~1900 and 3800 nts, respectively (Fig. 1A). Their sequences and those of the smaller rRNAs, however, are not unambiguously described in the literature (16, 21, 24–27) and also not in the Dictyostelium database. There, two transcripts for each rRNA are annotated that show variable sequences and ends, with the exception of the 5 S rRNA. Likely, these entries were generated by an automated gene prediction from the available sequences of the D. discoideum rRNA palindrome encoding the rRNAs (7), because their sizes do not coincide with the actual migration. In detail, the two 17 S rRNAs-1 and -2 in the database have a length of 2141 and 1871 nts, and the two annotated sequences of the 26 S rRNA start at different positions but are 3241 nts in length. There is no indication of the 2141 nts species that would correspond to the 17 S rRNA-1, although both 26 S rRNA annotations seem to be several hundred nucleotides short (Fig. 1A).

To analyze these discrepancies, we employed initially an RNase H assay. Here, we allowed antisense DNA oligonucleotides to hybridize to the 17 S and 26 S rRNAs within total RNA (Fig. 1B). The resulting DNA/RNA hybrids were then incu-
bated with the Moloney MLV-reverse transcriptase, an enzyme that displays RNase H activity. This is expected to cleave the RNA strand within any formed hybrid (28). The use of oligonucleotide p4 that binds within both annotated 17 S rRNA sequences leads to the appearance of a shorter fragment (Fig. 1C), as expected. A second cleavage product was not observed, and possibly, it was lost during the nucleic acid precipitation. No change in the RNA size was observed, when the RNA was incubated in the absence of oligonucleotides with the enzyme alone (data not shown). The binding site of oligonucleotide p5 lies within the annotated 17 S_rRNA-1 sequence but outside the 17 S_rRNA-2 sequence. The presence of p5 in the RNase H assay does not change the migration behavior of the 17 S rRNA (Fig. 1D), which indicates that the annotated 17 S_rRNA-1 sequence might not be present in the investigated RNA pool. This is also in line with the observation that no band of an apparent size of 2141 nts is found (Fig. 1A). Because oligonucleotide p12 can hybridize to the 26 S rRNA, it serves as a positive control. Its use leads to the appearance of two specific products of expected size (Fig. 1E). Finally, the use of oligonucleotide p9, which binds within the 3’ETS about 100 nts downstream of either annotated 26 S rRNA sequence, results in a size reduction of the 26 S rRNA species (Fig. 1F). These results indicate that the 26 S rRNA contain a segment that had been annotated as part of the 3’ETS.

Determination of the rRNA Ends by cRT-PCR—Because these data indicate that the rRNAs might be different from their annotations, we next set out to sequence the expressed rRNAs. To do so, we employed cRT-PCR, a very convenient method introduced by Grange and co-workers (29). In this, total RNA is subjected to ligation in cis, and the resulting circular RNA molecules function as templates for the generation of specific RT-PCR products (Fig. 2), which include both ends of a given RNA molecule.

First, we investigated the large rRNAs. Using specific primers in all steps of the cRT-PCR, we obtained clear reaction products for both the 17 S rRNA and 26 S rRNA (Fig. 3), and for the latter, we obtained a second apparently larger product, although at a considerably lower amount. The cRT-PCR product of the 17 S rRNA has a size of ~140 bp, which would be the expected size for a 17 S_rRNA-2-specific product (Fig. 3B). As before (Fig. 1), there is no indication for the presence of the 17 S_rRNA-1, for which a product of 408 bp would have been expected. For either of the annotated 26 S rRNA sequences, the

![Diagram of rRNA ends](image-url)

**FIGURE 2.** Principle of the cRT-PCR method used to determine the ends of RNA molecules. Total RNA is dephosphorylated by shrimp alkaline phosphatase (SAP) and then 5’-monophosphorylated by T4 polynucleotide kinase (T4 PNK). The resulting 5’-phosphate and the 3’-hydroxyl moieties (symbolized by up and down triangles, respectively) allow us to circularize individual RNA molecules by means of T4 RNA ligase. With the specific first strand primer 1, a cDNA product that contains the ligated ends of the RNA molecule can be generated using a reverse transcriptase. The 5’ to 3’ junction is then amplified by means of the specific primers 2 and 3 and Taq DNA polymerase, in a PCR, allowing for subsequent sequencing. Scheme was modified from Ref. 29.

![Diagram of rRNA amplification](image-url)

**FIGURE 3.** Amplification of the 17 S and 26 S rRNA ends by cRT-PCR. A, schematic representation of the rRNA transcription unit. The indicated oligonucleotides bind to either the RNA (reverse arrowheads) or its cDNA (forward arrowheads), respectively. All other description are as in Fig. 1B. Gel picture of the cRT-PCR product of the 17 S rRNA (B) and the 26 S rRNA (C) ends. The templates for the final PCRs were water (dH₂O), genomic DNA (gDNA), total RNA, circularized total RNA (cRNA), and sequence-specific cDNAs. For the 17 S rRNA, p4 was used as the first strand primer and p6 and p18 in the subsequent PCR, and for the 26 S rRNA p12 was used as the first strand primer and p28 and p32 in the PCR. M denotes the 100-bp plus DNA ladder (Fermentas), and DNA sizes are indicated in base pairs.
expected size of the cRT-PCR product would have been around 300 bp, when using the applied oligonucleotides. Instead, the main product has a size of ~800 bp. The absence of any shorter cRT-PCR product indicates that the mature 26 S rRNA is ~500 nts longer than annotated.

PCR products from circularized molecules featuring either rRNAs end were cloned, and a representative number of plasmids were sequenced. For the 17 S rRNAs, we obtained identical sequences of the 5’ and 3’ ends in six clones from two independent experiments. The results of these experiments show that the 17 S rRNA of D. discoideum consists predominantly of a single RNA species with a length of 1871 nts. It is different from the annotated sequences 17 S_rRNA-1 and -2 (supplemental Fig. 1) but identical to a sequence published based on an S1 nuclease protection assay (27). The 5’ end of the 17 S rRNA identified here was also confirmed independently by a sequencing approach on fractionated small RNAs from the Dictyostelium AX4 strain, where fragments starting with the same sequence were repeatedly observed (data not shown).

Similar to the situation for the 17 S rRNA, the sequencing of the cRT-PCR product of the 26 S rRNA also shows no heterogeneity at the 5’ or 3’ ends, as judged from sequencing six clones from two independent experiments. The 26 S rRNA does not coincide with either annotated sequence, neither at the 5’ end nor at the 3’ end. In particular, it contains an additional sequence of 500 nts at its 3’ end (supplemental Fig. 2). These data are in excellent agreement with the results of the RNase H assay and also with the apparent migration behavior during gel electrophoresis (Fig. 1).

The 5.8 S rRNA was analyzed next. We obtained a cRT-PCR product of about 180 bp (data not shown), which we subsequently cloned. Sequencing of six clones generated in two independent repetitions revealed a uniform 5.8 S rRNA sequence with a size of 162 nts. Despite the identical size, neither start nor end of the 5.8 S rRNA coincides with the automatically annotated sequences of 162 nts available in the Dictyostelium database (supplemental Fig. 3). The aforementioned sequencing approach confirms the 5’ end of the 5.8 S rRNA also for the AX4 strain, where the 3’ end, however, apparently shows some heterogeneity with molecules being 1, 2, 4 and once also 6 nucleotides shorter (data not shown). In most organisms, there is a long and a short form of the 5.8 S rRNA that vary in yeast by seven nucleotides at either end of the 5 S rRNA (data not shown), which again was cloned. An analysis of six sequenced plasmids from two independent repetitions shows the same sequence of the 5 S rRNA with a size of 112 nts. An alignment of these sequences and the encoding sequence of the rRNA palindrome (7) reveals, however, that only 105 nts can be unambiguously annotated (supplemental Fig. 4), although the remaining seven nucleotides can be either at the 5’ or the 3’ end. The 112-nucleotide sequence is 572 nts shorter than the automatically (and clearly erroneously) annotated sequences. It is still 7nts shorter than a sequence published earlier (25), which features the ambiguous stretch of seven nucleotides at either end of the 5 S rRNA. The latter sequence represents the 5 S rRNA of the NC-4 strain. Sequencing of the fractionated RNA pool shows that the AX4 strain,
which is a derivative of the NC-4 strain, also features this 119-nt-long 5 S rRNA sequence (data not shown). This indicates that there might be variation in the 5 S rRNA of different *D. discoideum* strains.

Summarizing this part, we could determine the sequences of all four ribosomal rRNAs in axenically grown *D. discoideum AX2* wild type cells by cRT-PCR. Our results indicate that there is no heterogeneity in the sequence or the length of these mature rRNAs, and they fully explain the apparent migration behavior in agarose gels, which we have observed at the start of this study.

**Identification of Common Precursor Molecules of 5.8 S and 26 S rRNAs** —The signals of subsequently performed Northern blot analyses on total RNA (T) and nucleus-enriched RNA (N) with rRNA-specific probes support the results from the cRT-PCR analysis that the mature rRNAs display homogeneous sequences (Fig. 5). However, the 26 S and 5.8 S rRNA-specific probes, which we used in the Northern analysis, both identified an additional RNA molecule with an apparent migration behavior above the 26 S rRNA. A signal on a comparable height can also be seen in the ethidium bromide stain of nucleus-enriched RNA but not in Northern blots that were performed with 17 S rRNA- or 5 S rRNA-specific probes. These observations led to the assumption that a so far unidentified RNA species exists, which contains the 26 S and 5.8 S rRNA, and thus could constitute a precursor molecule for these two rRNAs.

To characterize this potential precursor molecule, we again used cRT-PCR, however with different oligonucleotides. After circularization and cDNA synthesis using primer p12 for first strand synthesis, a 26 S rRNA (p28) and an ITSII-specific primer (p19) were used in the final PCR (Fig. 6A). The choice of these oligonucleotides excluded cRT-PCR products that would be derived from the mature 26 S or 5.8 S rRNAs. By this, we obtained cRT-PCR products with a size of ~850 bp and, additionally, more diffuse higher products (Fig. 6D), of which the former were cloned for subsequent sequencing. We sequenced 12 plasmids that were generated in two independent repetitions and determined the 5′ and 3′ ends of two potential precursor molecules with different sizes, which we termed 26 S pre1 and 26 S pre2. The larger molecule, 26 S pre2, contains at its 5′ end either 53 or 52 nucleotides of the ITS1, in three or four clones, respectively (supplemental Fig. 5A). The 5′ end of the smaller precursor, 26 S pre1, however, coincides with the previously determined 5′ end of the 5.8 S rRNA. The 3′ end of either type of precursor is heterogeneous in length; some have the same 3′ end as the 26 S rRNA; some are a few nucleotides longer, and two have an addition of a stretch of adenosines (supplemental Fig. 5B). These heterogeneous 3′ ends of both 26 S pre1 and 26 S pre2 point toward a 3′-exonucleolytic processing of these precursor molecules.

To assess if the 5.8 S and 26 S rRNAs are directly generated from these precursors or, alternatively, if additional processing intermediates can be observed, we performed a cRT-PCR with oligonucleotide p2 as first strand primer and p2 and p17 as PCR primers. In the PCR, the elongation time was reduced such that an amplification of products from either 26 S pre1 or 26 S pre2 was
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excluded. Under these conditions, we obtain two products with sizes of ~320 and 640 bp (Fig. 6C). They were cloned, and three independent clones derived from the 320-bp cRT-PCR product were sequenced. They all display the same uniform sequence, which apparently is derived from a 5.8 S rRNA-specific precursor with a size of 509 nts. A comparison of this RNA species, which we termed 5.8 S pre1, with the previously identified 5.8 S rRNA shows that both molecules have the same 5’ end (supplemental Fig. 6A). The additional sequence of 347 nts at the 3’ end of 5.8 S pre1 matches with the corresponding ITSII rDNA (supplemental Fig. 6B). The uniform 3’ end of this precursor is evidence to suggest that it might be derived from an endonucleolytic cleavage within 26 S pre1.

Two Precursors of the 17 S rRNA—We have also searched for precursor molecules of the 17 S rRNA. After circularization, a 17 S rRNA-specific cDNA was generated with oligonucleotide p4 as first strand primer. A predominant product of ~220 bp emerged in the subsequent PCR, which was performed with primers p6 and p7 (Fig. 6B). Upon cloning, 13 plasmids generated in two independent repetitions were sequenced, which revealed an identical core sequence of 1987 nts. An alignment of the 17 S rRNA revealed a nuclear localization of the 3’ end (supplemental Fig. 7). At the reaction temperature of 42 °C, primer p38 only is expected to bind, and p38 only if several adenosine residues were present at the 3’ end of precursor 17 S pre1. In the subsequent PCR, in which p38 and p18 were used, a product of the expected size of 160 bp was observed only in the p38-generated cDNA. These PCR fragments were sequenced and matched exactly to the expected stretch of the rDNA. Next, we analyzed whether this molecule localizes to the cytoplasm or to the nucleus. To this end, we isolated nuclear RNA and for comparison a nuclear depleted RNA fraction. RT-PCR on these two RNA fractions, using oligonucleotide p38 as first strand primer as described above, revealed a nuclear localization of the 3’-polyadenylated precursor 17 S pre1 (data not shown).

![FIGURE 6. Analysis of rRNA precursors by cRT-PCR.](image1)

![FIGURE 7. RT-PCR on the 17 S rRNA precursor.](image2)
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**FIGURE 8. Detection of the 17 S rRNA precursors from D. discoideum.** A, organization of the regions surrounding the 17 S rRNA. The position where the complementary DNA oligonucleotides p39, p36, p6, and p8 bind are shown by arrowheads. All other descriptions are as in Fig. 18 B. Northern blot analysis of rRNA species in total (T) and nucleus-enriched (N) RNA. Each lane contains 20 μg of the indicated RNA preparation, and the gel is either stained with ethidium bromide or subjected to Northern blot hybridization with the indicated radiolabeled oligonucleotides as sequence-specific probes. The migration of the 26 S and the 17 S rRNAs is indicated from the ethidium bromide staining. C, primer extension analysis using radiolabeled oligonucleotides p6 and p36. Reaction products were analyzed on denaturing polyacrylamide gels of 10% (left panel) and 5% (right panel) to allow for a good separation. M1 denotes the ultralow range marker and M2 the 100-bp plus DNA ladder (Fermentas). DNA sizes are indicated in base pairs. Note that the region between 400 and 500 bp is represented on both gels.

To assess the stability of precursor 17 S pre,1, we performed Northern blot analysis, on total RNA (T) and nucleus-enriched RNA (N) (Fig. 8). Using oligonucleotide probe p8, which is complementary to the 3’ end of the 17 S pre,1 within the ITSI sequence, surprisingly, we observed two signals. The lower signal corresponds to an RNA molecule somewhat larger than the 17 S RNA, as inferred from a comparison with an ethidium bromide-stained agarose gel, and its size matched that of the 17 S pre,1 precursor. Well above that, however, another signal was observed. To evaluate whether this might correspond to another larger precursor of the 17 S RNA, we next employed p36 and p39 that correspond to the 5’ and the 3’ end of the 5’ETS, respectively. Indeed, both probes picked up the same upper signal in Northern blot analyses (Fig. 8). From this, we conclude that a second precursor molecule of the 17 S rRNA exits, which we termed 17 S pre,2. Because probe p39 corresponds to the start site of the RNA polymerase I transcription unit (26), 17 S pre,2 appears to span the entire sequence from the 5’ETS up to the end of the 17 S pre,1 precursor.

**Absence of the Yeast A0 Processing Site in the D. discoideum rRNA Transcription Unit.** In S. cerevisiae, there is a well characterized processing site (A0) within the 5’ETS (35, 36), which, according to the experiments shown here, seems to be absent in D. discoideum. To confirm this, we performed primer extension analyses, using p36 and p6, which are complementary to the 3’ end of the 5’ETS and the 5’ end of the 17 S rRNA, respectively. From both primers, we obtained specific extension products. The use of p6 results in two extension products with sizes of about 110 and 900 nts (Fig. 7C). These signals correspond to the expected sizes for the 5’ ends of the 17 S and the 5’ETS. For p36, only one extension product was observed with a size of 800 nts, which again can be attributed to the 5’ end of the 5’ETS. Thus, neither cRT-PCR nor primer extension analysis provided evidence for a processing site in the 5’ETS of the D. discoideum rRNA transcription unit that would correspond to the yeast A0 site.

**Detection of the 37 S Primary Transcript.** Having shown that there are two precursors of the 17 S and 26 rRNAs, which are stable enough for detection, we next investigated if we could observe the 37 S primary transcript of the rRNA transcription unit that also has been seen by others (16, 21). To this end, we performed reverse transcription reactions using different first strand primers that were complementary to the 5’ end of ITSI or to the 5’ or 3’ end of the 26 S rRNA, respectively (Fig. 9). These cDNAs were then used as template for PCRs using primers that amplify a fragment corresponding to the 5’ETS, the ITSI, or part of the 26 S pre,1 molecule. For either cDNA, the same specific RT-PCR product is observed (Fig. 9). Because even for the cDNA generated with primer p15, which resides at the 3’ end of the 26 S rRNA, a specific product is obtained using the oligonucleotides p1 and p36 that match the 5’ETS (Fig. 9B), we propose the existence of an rRNA primary transcript. In view of the absence of a signal of this primary transcript in Northern blots, however, it likely is short lived.

**DISCUSSION**

In this study, we have determined the sequences of the four mature ribosomal RNAs present in an axenically grown D. discoideum AX2 strain. They do not display any sequence variation, indicating that the rDNA templates, which reside in extrachromosomal palindromes in the amoeba (7–9), are identical in their sequence, at least those that are actively transcribed. Our data fully explain the migration of the rRNA in agarose gels (Fig. 1). Based on the sequence information of the rDNA palindrome (7), our analysis further allowed us to adjust earlier studies on the D. discoideum rRNAs (16, 25, 27) that are in part derived from other strains. We show that cRT-PCR can be successfully employed on D. discoideum. As the method relies on
ends, however, we cannot exclude the possibility that, next to the precursors described here, additional ones exist that would not be picked up by this method. Using various RT-PCR setups, Northern blots, primer extension analysis, and sequencing, we have shown the existence of several precursors of the mature rRNAs that are generated from the RNA polymerase I-transcribed primary transcript. For these, we should like to propose a scheme describing consecutive processing events (Fig. 10). For comparison, we have included a sequence alignment of the \textit{D. discoideum} processing sites to the relevant sites in \textit{S. cerevisiae}. The primary transcript (37 S) is a molecule, which is stable enough for detection by RT-PCR (Fig. 9) or other techniques applied earlier (16, 21). From electron microscopy studies (24), however, it had been proposed, that the majority of precursors of the 17 S rRNA and the 5.8 S and 26 S rRNAs are already processed co-transcriptionally.

![Image of Ribosomal RNAs in Dictyostelium discoideum](image-url)
Ribosomal RNAs in Dictyostelium discoideum

...tionally. Because we do not detect the 37 S precursor in either of our Northern blots, it seems plausible that this indeed is the case. The more sensitive RT-PCR might pick up the smaller fraction of molecules that are processed only after the 37 S precursor has been released in the nucleolus (24).

The 37 S precursor is cleaved at three sites c1 to c3 that separate the 17 S contained within the 17 S precursor molecules from the bicistronic 26 S precursor species. In view of the heterogeneous 3’ end of the 26 S precursor (supplemental Fig. 5), c1 likely constitutes an exonucleolytic cleavage event, preventing a sequence comparison with the yeast site (Fig. 10D).

The 17 S precursor (Fig. 8) is processed at c4 to yield the mature 5’ end of the 17 S rRNA (supplemental Fig. 8); Northern blot analysis indicates the presence of a second stable processing intermediate that we termed 17 S pre1 (Fig. 8). From cRT-PCR analysis it appears that the majority of the 17 S pre1 Precursor molecules feature a nontemplated polyadenylated 3’ end (Fig. 7). Transcripts of RNA polymerase I are normally not expected to be polyadenylated. In recent years, however, several studies have shown that polyadenylation takes place in several species also beyond the realm of mRNA. For example, in S. cerevisiae (31), Schizosaccharomyces pombe (34), or Homo sapiens (37), polyadenylation marks aberrant or shortened rRNA transcripts for degradation. In analogy to this, the nuclear polyadenylated precursor 17 S pre1 that we observe would be marked for degradation, and only the nonadenylated sequence, which we identified once by cRT-PCR (supplemental Fig. 7), would give rise to the mature 17 S rRNA by means of cleavage at position c7 (Fig. 10B).

Nuclear polyadenylation does not, however, necessarily lead to the complete removal of the targeted RNA. For example, in S. cerevisiae is the nuclear exosome, a multiprotein complex displaying 3’-degradation activity, involved in the processing of snRNA precursors in dependence of their 3’-polyadenylation (38). Also for D. discoideum, snRNA precursors were detected, which showed a 3’ poly(A) tail with 20–30 residues on average (39).

Although the exosomal processing mechanism has been shown only for snRNAs so far, it is tempting to speculate that it might be operational on the polyadenylated precursor 17 S pre1 too. This would be an alternative to the degradation scenario described above, and it would require this molecule to localize to the nucleus. Here, the added adenosines would serve as signal for the removal of the partial ITS1 sequence that is still contained within precursor 17 S pre1, leading finally to the generation of the mature 17 S rRNA at position c7. This scenario might also serve to explain why barely any nonadenylated sequence is detected by cRT-PCR (supplemental Fig. 8), although there is a clear signal for precursor 17 S pre1 on Northern blot (Fig. 8).

A precursor molecule of the 26 S rRNA has been recognized earlier (16); however, that it contained the 5.8 S rRNA had not been realized. The alignment of sequences derived from the two bicistronic 26 S pre1 and 26 S pre2 precursors shows two distinct 5’ ends (supplemental Fig. 6). They correspond to that of the mature 5.8 S rRNA species or about a 50-nt longer species, respectively. It thus seems likely that the processing at position c5 represents an endonucleolytic cleavage event (Fig. 10C). The

3’ ends, however, display heterogeneous ends for either 26 S rRNA precursor, which corresponded to the mature 3’ end of the 26 S rRNA or sequences that were 3 or 5 nucleotides longer. Furthermore, in two clones, we observed short poly(A) tails that apparently were not encoded in the respective DNA (supplemental Fig. 6). Because these heterogeneous ends have been observed for both the 26 S pre1 and the 26 S pre2 precursors, it seems plausible that processing at position c6 can take place before or after that at position c5 (Fig. 10C), and in either case it might again involve a polyadenylation step. Next, cleavage at c8 generates the 5.8 S pre1 precursor (supplemental Fig. 6) and at c9 generates the mature 5’ end of the 26 S rRNA. Finally, the mature 5.8 S rRNA species is obtained by a processing event at position c10 (Fig. 10C).

The final step in the maturation of the 5.8 S rRNA in yeast is the trimming of the 3’ end by the RNA nuclease Ngl2p (40). In higher eukaryotes like Mus musculus and Caenorhabditis elegans, the same reaction is catalyzed by Eri-1 (41, 42). We have tested whether the 5.8 S rRNA of D. discoideum is extended in a knock-out strain of the homologous gene (DDB_0283113 in the Dictyostelium database); the strain was kindly provided by Benjamin Boesler and Wolfgang Nellen. Using the same cRT-PCR approach as done in the wild type strain AX2, we do not find any indication for extended sequences of the 5.8 S rRNA nor of the 17 S or the 26 S rRNAs that we also tested (data not shown).

At present, none of the enzymes that catalyze the processing of the 37 S rRNA precursor at the (at least) 10 cleavage points c1–c10 that our model predicts (Fig. 10) is known for the amoeba, and their identification will be a challenging task. It is, however, necessary to fill this model with life, and the identification of these enzymes will also allow us to verify, modify, or falsify the model according to the obtained results. Because the majority of enzymes and enzyme complexes involved in these processes are known for S. cerevisiae (17, 18), this can serve as a reference to initially identify the homologous proteins in the amoeba. Such enzymes would then have to be tested to assess whether they also adopt corresponding functions.

Overall, processing of the D. discoideum 37 S precursor appears to be similar to that of the 35 S precursor in yeast (17, 18). There are, however, also differences; in particular, the cleavage point is absent that would correspond to A0 site in S. cerevisiae, at which the rRNA of yeast is processed by the small subunit processosome (36). Furthermore, D. discoideum seems to contain one predominant 5.8 S rRNA species, compared with the two forms that are present in yeast and other organisms.

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