Precursors of Ribosomal RNA in the Cellular Slime Mold

Dictyostelium discoideum

ISOLATION AND CHARACTERIZATION*

Bambi Batts-Younge,† Nancy Maizels,§ and Harvey F. Lodish‡

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From †Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and §The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

The pathway of ribosomal RNA biogenesis in Dictyostelium discoideum has been defined through identification, isolation, and characterization of the rapidly labeled nuclear RNAs which are intermediates in the process. Comparison of the methylation patterns, base compositions, two-dimensional oligonucleotide maps, and hybridization properties of these intermediate RNAs with those of mature rRNAs has established clearly the precursor-product sequence relationships supporting the following scheme for rRNA production and processing:

\[ 37S \rightarrow p_{25S} (28S) \rightarrow 25S \rightarrow p_{17S} (21S) \rightarrow 17S \]

The relationship of the 37 S RNA of Dictyostelium to primary rRNA transcripts of prokaryotes and other eukaryotes is discussed.

In addition to further cleavage steps, post-transcriptional modification of rRNA includes a number of site-specific methylations. In both prokaryotes and eukaryotes, at least some of the methylations occur early in the processing scheme; thus, methylation is a characteristic feature of all rRNAs and most of their precursors (9). However, almost all the eukaryotic rRNA methyl groups are ribose substitutions, and are introduced early, at the primary transcript stage; while bacterial methylations are mostly base substitutions, and many do not appear until the very last stages of ribosome maturation (1, 6). Eukaryotes have thus diverged from prokaryotes in these and other aspects of the rRNA synthetic program. Study of this program in a variety of lower eukaryotes should provide evidence about the origins and mechanisms of the divergence.

Dictyostelium discoideum is a lower eukaryote of particular interest because of its simple, well defined developmental program. In the vegetative phase of its life cycle, a homogeneous population of single celled amoebae grows and multiplies. When cells are deprived of nutrients and placed on a solid surface, they synchronously aggregate into multicellular fruiting bodies, and differentiate into two new cell types: stalk cells and spore cells. Distinctive alterations in the pattern of rRNA synthesis and processing accompany the other biochemical and morphogenetic changes in the developmental sequence (10-12). One example is a marked decrease in the rate of transport of total RNA out of the nucleus in developing compared to vegetative cells, and an associated accumulation of those RNAs thought to be rRNA precursors (12). We have undertaken a thorough characterization of all the intermediates in the maturation of Dictyostelium rRNA. Such analysis should provide the framework for future investigations into the nature and causes of altered rRNA production during development.

Previous research has suggested that the mature rRNAs of Dictyostelium are ultimately derived from a common, large molecular weight precursor, via two intermediate species slightly larger than the final rRNA molecules (13). Evidence for this pathway has depended solely on examination of the kinetics of incorporation of radioactive precursors into total cellular or nuclear RNA. Attempts to chase radioactive label from short lived intermediates into mature rRNAs have failed to demonstrate clearly a precursor-product relationship, presumably because of problems in diluting the large pools of RNA precursors. We describe in this paper the isolation of...
each of the putative precursor rRNAs as a discrete species, and demonstrate definitively the precursor-product sequence relationship between them and mature rRNAs.

**EXPERIMENTAL PROCEDURES**

**Materials—Radioactive compounds were purchased from New England Nuclear Corp.** $^{32}$P, was carrier-free, and [methyl-3H]methionine had a specific activity of 11.5 Ci/mmol.

Diethylpyrocarbonate, packed under nitrogen, was obtained from Sigma; after a bottle had been opened, it was used for only 2 weeks before being discarded. Sodium dodecyl sulfate was recrystallized twice from 95% ethanol. Phenol was redistilled. *Escherichia coli* K-12 tRNA, used for carrier, was purchased from Schwarz/Mann, extracted three times with phenol, and bound to and eluted from a DEAE-cellulose column.

$^{32}$P-labeled poliovirus virion RNA was generously provided by Albert Reber of Yale University; $^{32}$P-labeled 28 S and 18 S marker RNAs were prepared by phenol extraction of Chinese hamster ovary cells.

**Medium and Buffers—Axenic growth medium (Medium MES-HLS)** contains, per liter, 5 g of Difco yeast extract, 10 g of Difco peptone, 10 g of glucose, and 6 mM Mes, at pH 6.8.

Starvation buffer (Buffer MES-PDF) consists of 20 mM KCl, 5 mM MgSO$_4$, 0.3 g/liter of streptomycin sulfate, and 7 mM Mes, at pH 6.5. HMK buffer is 40 mM MgCl$_2$, 20 mM KCl, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, adjusted to pH 7.5 with NH$_4$OH.

**Cytoplasmic RNA was recovered by centrifugation at 10,000**

**Growth, Starvation, and Radioactive Labeling**

Cells—Vegetative strain AX3 cells were grown axenically to a density of 2 x 10$^7$ cells/ml. Cells were then transferred to buffered filter paper or shaken at 23°C in buffered suspension. Starvation conditions, either (a) on buffered filter paper or (b) in cold Buffer MES-PDF, and resuspended at 10$^7$/ml in Buffer MES-PDF, with shaking, at 25°C. Starved in this manner mimic the biochemical changes of preaggregation stage developing cells, but never progress beyond the point of forming loose cell clumps.*

**Cell Fractionation and RNA Extraction—Cells** (2 x 10$^8$) were collected by centrifugation and washed with ice-cold Buffer MES-PDF. The cell pellet was frozen in dry ice/ethanol for 10 min, and then washed with 4 ml of Cemulso NPT-12 lysis buffer, followed by resuspension in 5 ml of fresh lysis buffer, followed by recentrifugation. The final nuclear pellet was resuspended in 5 ml of HMK buffer for preparation of deproteinization.

For centrifugation, each preparation of nuclei or polysomes was mixed with 0.3% (w/v) sodium dodecyl sulfate and then 2% (v/v) in diethylpyrocarbonate. Over 10 s after addition of the diethylpyrocarbonate, 1 volume of water-saturated phenol was mixed with the sample. Then 0.1 volume of 2.5 M sodium acetate, pH 7.3, was added, followed by 1 volume of chloroform:isoamyl alcohol (95:4.5). The mixture was agitated on a vortex mixer for 3 min, then centrifuged at 4,000 x g for 7 min. The two phases separated by the centrifugation were each re-extracted separately: the upper aqueous phase plus interphase with 2 volumes of phenol:chloroform:isoamyl alcohol (96:4:2), and the lower organic phase with 2 ml of HMK buffer containing 0.2 M sodium acetate, pH 7.3. Both samples were agitated and centrifuged as above. The first (re-extracted) organic phase was discarded. The second organic phase was removed from under the sample aqueous phase, and was re-extracted with the same 2-ml HMK buffer phase used for the first organic phase. After this second aqueous phase re-extraction, the two aqueous phases, together with the phenol:chloroform:isoamyl alcohol mixture, were pooled and re-extracted twice with 2.5 volumes of chloroform:isoamyl alcohol mixture (96:4) alone. The RNA in the final aqueous phase was precipitated with 2 volumes of ethanol. Coplysmic RNA was recovered by centrifugation at 10,000 x g for 10 min; nuclear RNA at 250,000 x g overnight.

Ribosomal RNAs were separated from heterogeneous, poly(A)-containing RNAs by oligo(dT)-cellulose (Collaborative Research) chromatography. RNA pellets were dissolved in 0.5 to 1.0 ml of oligo(dT)-cellulose binding buffer and applied to a 0.1 to 0.25-g column of oligo(dT)-cellulose equilibrated in the same buffered agarose solution, but without glycerol. The column was washed with 10 volumes of binding buffer; the flow-through solution contained most of the tRNA. RNA bound to the column was collected when the column was washed with oligo(dT)-cellulose elution buffer; the eluate fraction was made 0.4 M in sodium acetate, pH 5.2. Both the total and the eluted fractions were harvested by precipitation with ethanol as described above.

**Agarose Gel Electrophoresis of RNA**—The gel system used was a modification of that of Sharp et al. (16). The 1.4 to 1.5% (w/v) agarose (Calbiochem or Sigma electrophoresis grade) was melted in E buffer (50:48:2) and the lower organic phase with 2 ml of HMK buffer containing 0.2 M sodium acetate, pH 7.3. Both samples were agitated and centrifuged as above. RNA was precipitated with ethanol as described above.

**Gel Electrophoresis of Cytoplasmic RNAs**—Cytoplasmic polyribosomal RNA which did not bind to oligo(dT)-cellulose was prepared from cells starved in suspension for 10 h and labeled with 60 mCi of $^{32}$P, during the entire starvation period. Mature 2S, 17 S, and 4 to 5 S RNAs were separated by centrifugation through 34 ml 15 to 30% (w/v) linear sucrose gradients in Buffer A. Centrifugation was for 17 h at

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1. Buffer MES-PDF is a phosphate-free variant of pad diluting fluid.
2. Buffer PDF consists of 13 mM KCl, 5 mM MgSO$_4$, 0.75 mM CaCl$_2$, 0.3 g/liter of streptomycin sulfate, 22 mM NaH$_2$PO$_4$, and 11 mM Na$_2$HPO$_4$.
23° and 25,000 rpm in a Beckman SW 21 rotor. The 5 S RNA was purified from material near the top of the gradient by procedures described elsewhere (23). The 23 S and 25 S RNA fractions were then sedimented separately on 15 to 30% gradients, and the peak fractions were recovered. The purified RNA pools were concentrated by centrifugation with 2 volumes of ethanol, followed by centrifugation at 10,000 x g for 15 min. Each RNA pellet was dissolved in 0.5 ml of water and layered onto a 5-ml Sephadex G-25 M column in water. RNA was eluted from the column with water, and the salt-free void volume fractions were collected. The specific activity of the purified mature rRNA was 1,500,000 cpm/μg.

**Purification of Nuclear rRNAs**—Nuclear RNA was prepared from cells starved in suspension for 1 h and labeled at the same time with 60 mCi of [α-32P]ATP. Usually further purification steps were carried out on RNA that flowed through oligo(dT)-cellulose, but 37 S RNA could also be purified from material that bound to the column under high salt conditions. In either case, nuclear RNAs were fractionated by sedimentation through 11 ml 15 to 30% (v/v) linear sucrose gradients in Buffer A; centrifugation was for 7.25 h at 23° and 37,000 rpm in a Beckman SW41 rotor. Fractions from the radioactive peaks sedimenting at roughly 21 S and 28 S were pooled separately; all material sedimenting faster than 31 S was collected in a third pool. Each RNA pool was precipitated with ethanol. The RNA pellets were dissolved in gel sample solution, and fractionated further by electrophoresis on a cellulose acetate strip (3 by 55 cm; Schleicher and Schuell). Regions of the gels which contained bands of 37 S RNA, ~25 S RNA, or ~17 S RNA were excised, and each sample was forced through a disposable syringe (20-gauge needle) into a centrifuge tube. A volume of gel elution buffer at least twice the gel volume was added, and the sample was mixed vigorously at room temperature for 3 min. Large gel particles were removed by centrifugation at 4,000 to 10,000 x g for 5 to 10 min, and the supernatant was passed over glass wool to filter out remaining particles. Extraction of the crushed gel was repeated one to three times, until 90% of the radioactivity had been eluted. All extraction supernatants for a given sample were pooled, and the RNA was twice precipitated with ethanol. The individual RNA species were finally purified by traces of contaminating RNA, or gel residues by sedimentation in 15 to 30% sucrose gradients identical to those used for initial nuclear RNA fractionation, except that the centrifugation of 17 S RNA was for 10.25 h. As each pure RNA represented less than 1 μg of material, 10 to 15 μg of carrier RNA were added before the RNA was concentrated by ethanol precipitation. To ensure that the RNA was free of sodium dodecyl sulfate and other salts, it was precipitated from ethanol at least three times. The final pellets were dried and resuspended in small volumes (20 to 75 μl) of water.

**Fingerprinting of RNase A or T1 Digests of RNAs**—The homochromatography fingerprinting method used was essentially modified from the procedure described by Barrell (19). Each [32P]-labeled RNA digestion sample was mixed with sufficient cold carrier RNA to yield 20 μg total of RNA. The sample was dried in a vacuum desiccator and resuspended in 2.5 μl of either 200 μg/ml of RNase T1 (Calbiochem) or 200 μg/ml of RNase A (Worthington), each in 10 mM Tris-HCl, pH 7.5. Digestion was for 30 min at 37 °C. Digested RNA nucleotides were separated in the first dimension by pH 3.5 isoelectrofocusing on a cellulose acetate strip (3 by 55 cm; Schleicher and Schuell) at 7000 V for 30 min. Separation in the second dimension was by homochromatography on a plastic-backed thin layer plate (20 by 20 by 40 cm) of polyethyleneimine-cellulose (Brinkmann Instruments); the homochromatography used was type C10, digested for 10 min with 1 n KOH.

**Hybridization of RNAs with Fractionated EcoRI Restriction Fragments of DNA**—Procedures for isolation of Dictyostelium discoidum nuclear DNA and digestion with EcoRI restriction endonuclease have been described previously (20). After EcoRI digestion, the DNA sample was adjusted to 12% (v/v) glycerol, mixed with xylene cyanol FF and bromphenol blue tracking dyes, and layered onto a 15% (v/v) agarose slab gel of the same type as used for RNA fractionations (above). Electrophoresis was at 10 V for 18 h, until the xylene cyanol dye had moved 23 S and 25 S RNA. Gels were stained for 0.5 h in 0.5 μg/ml of ethidium bromide, and then illuminated with a short wave ultraviolet lamp (Ultra-Violet Products) for photography through a red filter on Polaroid 107 positive film (16). Transfer of the fractionated DNA pattern to nitrocellulose filters (Schleicher and Schuell) was by the procedure of Southern (21). DNA filters were wet uniformly with the minimum possible volume of a mixture containing 50% (v/v) formamide (M/C-B), 5 x SSC, and 3,000 to 80,000 cpm of 32P-labeled RNA; each filter was sealed in Saran Wrap and incubated for 12 to 15 h at 42°C. In preparation for autoradiography, the filters were washed several times in 5 x SSC, incubated for several hours at 42°C in a large volume of 50% formamide/5 x SSC, and then washed extensively in 5 x SSC.

**RESULTS**

In order to isolate and identify short lived intermediates in the program of ribosomal RNA maturation, we have taken advantage of the decreased rate of nuclear RNA processing which is a special property of developing cells of Dictyostelium. As a result, it has been possible for us to obtain accumulated supplies of rRNA precursors without disturbing normal physiological processes by the introduction of drugs or mutations. For these experiments, we have used cells starved in suspension, as well as developing cells, which are starved on filters (see "Experimental Procedures"). We can detect no differences in the overall pattern of nuclear RNAs from cells starved in suspension compared to cells starved on filters, but it is much easier to label RNA in suspension cells.

Initially, two criteria were used to identify possible rRNA precursors in the nucleus: rRNA-related species should be multiply methylated, and should not bind to oligo(dT)-cellulose columns. [methyl-3H] and [32P]-labeled RNAs, extracted from the nuclei of aggregation stage developing cells after a labeling period of 3 h, were passed over oligo(dT)-cellulose, and the flow-through fraction was analyzed by agarose gel electrophoresis (Fig. 1). The large molecular weight RNA species were resolved clearly on the gel into five discrete species. Two of these species co-migrate with mature, cytoplasmic 25 S or 17 S rRNA. Of the remaining RNA peaks, two are labeled "25 S" and "17 S," in accordance with their presumed roles as immediate transcripts of RNase A, RNase T1, and RNase T2 were performed as described by Rose (18). Digests were spotted on sheets of Whatman No. 3MM paper and subjected to pH 3.5 isoelectrofocusing at 40 V/cm for 50 to 60 min, until xylene cyanol FF dye moved 11 to 12 cm from the origin (19). The four ribonucleoside monophosphates were detected by autoradiography, excised from the paper, and assayed for radioactivity in a toluene-based scintillation fluid.

![Fig. 1. Profile of developing cell nuclear RNAs which are methylated and which do not bind to oligo(dT)-cellulose.](http://www.jbc.org/)

* B. Batts-Youn and H. F. Lodish, manuscript in preparation.
dictate precursors to 25 S RNA and 17 S RNA, respectively. The last and largest RNA is most likely candidate for the primary rRNA transcription product. We designate this species "37 S" RNA, in accordance with the terminology used to describe other primary transcript rRNAs; however, by this title we mean to indicate only an approximate value for the sedimentation coefficient of the RNA (see below). Although all of the discrete nuclear RNAs can incorporate substantial quantities of label from [methyl-3H]methionine, as would be expected of ribosomal RNAs and their precursors, there is a marked difference in the ratio of H to 32P in "precursor" compared to 25 S and 17 S RNAs, demonstrating that the precursors are not aggregates or conformational variants of the mature rRNAs. Thus, the 3H/32P ratio for both 25 S and 17 S RNA is approximately 0.8 (1 ×), while the comparable ratio is 1.55 (2 ×) for p25 S and p17 S RNA, and 3.6 (4 ×) for 37 S RNA.

We have examined nuclear RNA profiles from developing and suspension starved cells labeled with 32P, for periods ranging from 10 min to 10 h (data not shown). In no case do we detect any discrete large molecular weight RNAs other than the five described above. The radioactive discrete nuclear RNAs isolated from cells labeled for short periods consist almost exclusively of the putative precursor species; only after an hour does label begin to appear in mature rRNAs, and these then are probably cytoplasmic contaminants. The purity of labeled "precursor" RNAs in the nuclei of cells labeled for only 1 h is illustrated by the gel profile of Fig. 2A.

In order to obtain profiles so clean as that illustrated in Fig. 2A, it is essential first to pass the RNA preparation over an oligo(dT)-cellulose column in high salt binding buffer, and material which flowed through the column under these conditions was collected. The RNA which had bound to the column was then recovered by elution with low salt elution buffer (see "Experimental Procedures"). Aliquots of each RNA fraction were analyzed by agarose gel electrophoresis. Cells were starved in suspension (see "Experimental Procedures"), and were labeled for 1 h with 60 mCi of Tricarb, immediately after the onset of starvation. At the end of the 1 h labeling period, cells were harvested and nuclear RNA was extracted from them. Individual RNA species were purified from total nuclear RNA as described in the text. In the last step, RNA eluted from an oligo(dT)-cellulose column was suspended in 0.25 ml of 0.05 M Tris-HCl, pH 7.5, 0.01 M EDTA, 0.05 M NaCl, and 0.001 M Na2HPO4, quick-cooled, and diluted to 0.25 ml with Buffer A. All samples were immediately layered onto 11 ml of 15 to 30% sucrose gradients and were purified by sedimentation through the gradients for 10.25 h (A) or 7.25 h (B to D). The Cerenkov radiation of each gradient fraction was measured directly (17). The positions of markers of 35 S poliovirus RNA, Chinese hamster ovary cell rRNA, and Dictyostelium cytoplasmic (25 S + 17 S) RNA were obtained from parallel gradients. Peak fractions from sample gradients were pooled as indicated by the bars. A, p17 S RNA; B, p25 S RNA; C, 37 S RNA; D, another preparation of 37 S RNA, after formamide denaturation.

![Fig. 2. Profiles of nuclear RNAs from suspension starved cells labeled with 32P, for only 1 h: fractionation by oligo(dT)-cellulose column chromatography. Cells were starved in suspension (see "Experimental Procedures").](#)

![Fig. 3. Sedimentation profiles of purified discrete nuclear RNAs.](#)

**Table 1**

**Base compositions of discrete nuclear RNA species compared to mature rRNAs**

<table>
<thead>
<tr>
<th>RNA sample</th>
<th>CMP</th>
<th>AMP</th>
<th>GMP</th>
<th>UMP</th>
<th>G + C</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 S RNA</td>
<td>18.5</td>
<td>28.5</td>
<td>24.2</td>
<td>29.0</td>
<td>42.5</td>
</tr>
<tr>
<td>p17 S RNA</td>
<td>19.0</td>
<td>30.3</td>
<td>22.0</td>
<td>28.6</td>
<td>41.1</td>
</tr>
<tr>
<td>25 S RNA</td>
<td>17.5</td>
<td>28.5</td>
<td>25.6</td>
<td>28.1</td>
<td>43.4</td>
</tr>
<tr>
<td>p25 S RNA</td>
<td>18.0</td>
<td>28.8</td>
<td>24.1</td>
<td>29.2</td>
<td>42.1</td>
</tr>
<tr>
<td>37 S RNA</td>
<td>17.9</td>
<td>29.8</td>
<td>22.1</td>
<td>29.3</td>
<td>40.9</td>
</tr>
</tbody>
</table>
oligo(dT)-cellulose column under such conditions that heterogeneous, poly(A)-containing RNAs are retained on the column. Fig. 2B indicates the composition of the material that binds to the column when salt concentration is high. There is considerable nonspecific sticking of the putative rRNA precursors to the oligo(dT)-cellulose; however, there does not appear to be preferential binding of any one of these species relative to another. Most of the bound radioactive material is heterogeneous, and presumably represents poly(A)-containing message-like RNAs (22). A large portion of the heterogeneous RNA migrates only slightly faster than p17 S RNA. Roughly 30 to 40% of total nuclear RNA binds to oligo(dT)-cellulose under the conditions used here. Thus in unfractionated nuclear RNA, heterogeneous material would obscure the profile of distinct species, particularly in the region of 17 S and p17 S RNA. This problem has made it impossible to determine from previous studies whether or not the proposed rRNA species were intact, discrete species.

Fig. 3 demonstrates that pure, discrete precursor rRNAs can in fact be isolated from nuclear RNA preparations comparable to that shown in Fig. 2A. The purification procedure is detailed under “Experimental Procedures” and involves fractionation of the individual RNA species on two sucrose gradients and an agarose gel. A to C of Fig. 3 represent characteristic profiles of the final purification gradients for p17 S RNA, p25 S RNA, and 37 S RNA. D provides additional evidence that 37 S RNA is not simply an aggregate of smaller RNAs. A preparation of 37 S RNA was heated at 65° in 90% formamide before being layered onto the final sucrose gradient. Under these denaturing conditions, the RNA did not dissociate into smaller RNA species (Fig. 3D). Peak fractions from the gradients were pooled as indicated in the figure, and were concentrated by precipitation with ethanol. From the mobility of sample peaks relative to marker RNAs on such gradients, we can estimate the size of each discrete nuclear RNA; thus, 37 S RNA actually has a sedimentation coefficient somewhere be-

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**Fig. 4.** Autoradiograms and tracings of two-dimensional separations of oligonucleotides produced by complete RNase A digestion of p25 S, 25 S, p17 S, and 17 S RNAs. First dimension separation, on cellulose acetate, pH 3.5, is based mainly on oligonucleotide charge; while second dimension homochromatography fractionates mainly on the basis of size, with smaller oligonucleotides moving toward the top of the plate (19). The tracings are composite of the fingerprints for a precursor rRNA and the corresponding mature rRNA. Open circles represent those oligonucleotides common to both; filled circles, those unique to the precursor species; and cross-hatched circles, those found exclusively, or in far greater quantity, in the mature rRNA. Oligonucleotides outlined in dots are faint, but reproducible in many fingerprints. Most of the faint oligonucleotides near the top of the fingerprints are not reproducible, and appear more frequently when the batch of digesting enzyme is old; they have therefore been omitted from the fingerprint tracings. The regions outlined with a dashed line represent characteristic oligonucleotide groupings that distinguish the p25 S-25 S RNA pair from the p17 S-17 S RNA pair.
between 36 S and 38 S, and the sedimentation coefficients for p25 S and p17 S RNAs are approximately 28 S and 21 S, respectively.

**Base Compositions** — The nuclear genome of *Dictyostelium discoideum* has a low, 22 to 23% G + C content (23-25), as does the total mRNA (26). In marked contrast is the 42 to 44% G + C content of mature ribosomal RNA (26) (see also Table I). As would be predicted for ribosomal RNA precursors, 37 S RNA, p25 S RNA, and p17 S RNA do not differ significantly in base composition from mature ribosomal RNAs (Table I).

**Oligonucleotide Fingerprint Analysis** — More compelling evidence for rRNA nucleotide sequence relationships has been obtained from examination of oligonucleotide map "fingerprints" of the RNase A digestion products of p25 S and p17 S RNAs, compared to equivalent fingerprints of the mature rRNAs (Fig. 4). Because RNase A recognizes both cytidine and uridine residues as cleavage sites, a large number of small oligonucleotides are generated by its action; such small fragments are expected to be distributed commonly among RNA molecules as large as rRNAs. Thus the upper portions of all the RNase A oligonucleotide maps are almost identical, even for such relatively unrelated species as 17 S RNA and 25 S RNA. However, in the lower regions, where large unique oligonucleotides are located, the fingerprints for 17 S RNA and...
25 S RNA differ significantly; some of the distinguishing oligonucleotide groupings are outlined in the fingerprint tracings. The fingerprints and tracings of Fig. 4 demonstrate clearly that all the unique oligonucleotides characteristic of 25 S RNA are also found in p25 S RNA. In fact, except for four extra large oligonucleotides in p25 S RNA, the fingerprints of p25 S RNA and 25 S RNA appear identical. Essentially the same type of relationship is seen between the fingerprints of p17 S RNA and 17 S RNA, although 17 S RNA contains one distinctive oligonucleotide, probably representing one end of the intact RNA, which is not found in p17 S RNA.

T1 RNase, with a greater degree of specificity than RNase A, cleaves next to guanosine residues only. Consequently, a T1 RNase digest contains many more large, distinctive oligonucleotides than does an RNase A digest. Fig. 5 illustrates the many sequences distinguishing 17 S RNA from 25 S RNA which are revealed in T1 oligonucleotide maps. The striking correlation of T1 oligonucleotide patterns in p25 S RNA compared to 25 S RNA, and in p17 S RNA compared to 17 S RNA (fingerprints and tracings of Fig. 5), therefore provides strong substantiating evidence for the precursor-product relationships suggested by the RNase A fingerprints. As is demonstrated by the tracings of Figs. 4 and 5, the RNase T1 fingerprint also exposes more clearly than RNase A fingerprints those extra oligonucleotides which distinguish each precursor from its mature rRNA counterpart.

Hybridization with EcoRI Fragments Which Code for rRNA—When Dictyostelium nuclear DNA is digested with EcoRI restriction endonuclease and is fractionated by agrose gel electrophoresis, nine discrete fragment bands, representing reiterated DNA sequences, are resolved above a background of heterogeneous fragments. One of these DNA bands (actually a doublet) hybridizes specifically with 25 S RNA; a second band hybridizes with 17 S rRNA; and a third band hybridizes with both rRNAs (20, 27). We have used these rRNA-specific restriction fragments as probes for the detection of rRNA sequences in proposed precursor rRNAs.

For the hybridization analysis, total nuclear DNA was digested with EcoRI, and the digest was fractionated on an agarose slab gel as described under "Experimental Procedures." The entire fractionated DNA pattern was then transferred from the gel to a nitrocellulose membrane by the procedure devised by Southern (21). 32P-labeled 5 S, 17 S, p17 S, 25 S, and 37 S RNAs were allowed to hybridize with the DNA on parallel strips of the nitrocellulose filter, each derived from one slot of the same gel. As shown in Fig. 6, the p25 S RNA hybridizes specifically to those EcoRI DNA restriction fragments complementary to 25 S RNA; similarly, the pattern of p17 S RNA hybridization is identical with that for 17 S RNA. Purified 37 S RNA anneals with all three of the rRNA-specific restriction fragment bands. Thus, this large RNA species contains the proper sequence complement required for a common precursor to 25 S and 17 S rRNAs.

DISCUSSION

We have characterized three discrete, rapidly labeled RNA species, all extensively methylated, from the nuclei of starved and developing Dictyostelium discoideum. These RNAs correspond in mobility and methylation properties to very transient radioactive species observed by Iwabuchi et al. (13) in sucrose gradient profiles of whole cell RNA from vegetative NC-4 Dictyostelium (NC-4 is parent to the axenic strain we have used). We assume that the comparable vegetative and developing cell RNA intermediates are the same. The one possible exception is a 30 S RNA intermediate, observed as a shoulder in Iwabuchi's gradients of vegetative RNA, which we do not detect in high resolution agarose gel profiles of nuclear RNA from starved or developing cells. Kinetics of RNA labeling in the vegetative cells suggested that the short lived intermediates were probably rRNA precursors. We have exploited the special properties of starved and developing cells to demonstrate further that each intermediate can be isolated as a discrete species, uncontaminated by heterogeneous material, incomplete chains, or aggregates of smaller rRNAs. It has not been possible to establish for Dictyostelium an effective pulse-chase procedure for demonstrating clearly the flow of label from precursor to product RNAs (13). We have instead applied fingerprinting and hybridization techniques, in order to obtain unequivocal evidence for the derivation of mature rRNAs from the sequences of the proposed precursors.

The evidence presented in this paper suggests that the 25 S and 17 S rRNA genes in Dictyostelium are associated in a large common transcription unit, which produces a 37 S RNA primary transcription product that includes sequences of both 25 S and 17 S mature rRNAs. Cleavage of the primary transcript yields the two immediate precursors of mature rRNA.
p25 S RNA, a molecule containing a small amount of transcribed spacer linked with the precise sequence composition of mature 25 S RNA, and p17 S RNA, which is related in much the same way to mature 17 S RNA. Mapping of restriction fragments produced from the rDNA of Dictyostelium demonstrates a very close association of the 25 S and 17 S RNA genes (20) (Fig. 7), and also provides some evidence that these genes lie on the same DNA strand. Therefore, the organization of the rDNA is at least consistent with production of a single primary transcript rRNA encompassing sequences programmed by both rRNA genes.

Moreover, by combining data from the rDNA restriction fragment map with our evidence about the properties of 37 S RNA, we can eliminate the possibility that this RNA actually consists of two 36 to 38 S RNA populations, one containing 25 S RNA, we can eliminate the possibility that this RNA actually consists of two 36 to 38 S RNA populations, one containing 25 S RNA, and the other 17 S RNA sequences, in each case linked to large stretches of transcribed spacer. We have shown that 37 S RNA hybridization is restricted to the same EcoR1 fragments which anneal with the mature rRNAs. Mobility of 37 S RNA relative to markers on gradients and gels, and analogy with the 36 to 38 S primary rRNA transcripts of other lower eukaryotes (28-30), allow us to set a lower limit for the length of Dictyostelium's primary rRNA transcript at approximately 8,000 nucleotides. Then if there were two independently transcribed 37 S RNAs, they would be coded by, and also provide evidence that these genes lie on the same DNA strand. Therefore, the organization of the rDNA is at least consistent with production of a single primary transcript rRNA encompassing sequences programmed by both rRNA genes.

In Fig. 7, the 5 S RNA gene is located somewhere in a 7000 base pair fragment (R1 7) which is neighbor to the terminal 25 S RNA gene fragment (20). What remains to be resolved is whether the primary rRNA transcription unit in Dictyostelium extends to include 5 S RNA sequences. The absence of obvious hybridization of 37 S RNA with the R1 7 fragment band (Fig. 6) argues against this possibility. However, the gene coding for 5 S RNA might be located at the very end of the R1 7 fragment, immediately adjacent to the rRNA genes. In such a case, 37 S RNA could include 5 S RNA with the addition of no more than 120 nucleotides, representing less than 2% of the total RNA length, in sequences complementary to the R1 7 fragment. Because extremely small quantities of radioactive purified 37 S RNA were available for these experiments, it is quite possible that hybridization of 37 S RNA to such a small piece of R1 7 would not be detected. Substantial evidence that 5 S RNA is not transcribed as part of a precursor common to 25 S and 17 S rRNAs has been obtained very recently. We can demonstrate that both 5 S RNA and p17 S RNA are initiated with a 5'-triphosphate residue in Dictyostelium. It should be of interest to characterize the relationship of 5 S RNA to primary rRNA transcript in a variety of lower eukaryotes, since the discovery of aspects of rRNA synthesis characteristic to intermediate level organisms can provide some insight into molecular mechanisms of evolution.

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Dictyostelium rRNA Precursors

Precursors of ribosomal RNA in the cellular slime mold Dictyostelium discoideum.
Isolation and characterization.
B Batts-Young, N Maizels and H F Lodish


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