Ribosomal Ribonucleic Acid Repeat Unit of *Acanthamoeba castellanii*: Cloning and Restriction Endonuclease Map†

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ABSTRACT: The repeat unit coding for the precursor to 18S, 5.8S, and 26S ribosomal ribonucleic acids (rRNAs) has been cloned from the free-living soil amoeba *Acanthamoeba castellanii*. The cloned deoxyribonucleic acid (DNA) was mapped with 11 restriction endonucleases and by R-loop mapping. The entire repeat unit is 12 kbp (kilobase pairs) in length and contains sites for *Eco*RI, *Sma*I, *Bgl*II, *Sst*I, *Bam*HI, *Pst*I, *Kpn*I, *Hin*dIII, and *Xba*I but not for *Xho*I or *Sal*I. All of the repeat units in the nuclear DNA appear to be identical, and no introns were detected. However, the regions which code for the two RNAs which comprise the 26S RNA are separated by a gap of approximately 200 base pairs. Unlike some other lower eukaryotes, the 5S RNA gene is not linked to this repeat unit. A fragment of the repeat unit which contains the initiation sequence of the putative precursor has been subcloned into pBR322 for use in in vitro transcription studies.

Acanthamoeba castellanii is a free-living protozoan which undergoes a transition from an amoeba to a dormant cyst when faced with starvation. During differentiation, the activities of several genes are developmentally regulated; foremost among these is the ribosomal ribonucleic acid (rRNA) transcription unit. In the vegetative cell (trophozoite), 18S + 26S RNAs represent 75% of pulse-labeled transcripts; by 10 h after experimental induction of synchronous encystment, these RNAs are no longer synthesized (Stevens & Pachler, 1973). Trivial explanations for this phenomenon have been ruled out: the number of DNA-dependent RNA polymerase I molecules in each cell remains constant during differentiation (Detke & Paule, 1975), and the catalytic properties of the enzyme do not vary. RNA polymerase I purified from trophozoites and cysts possesses the identical complement of polypeptide subunits (Detke & Paule, 1976). This latter result has been confirmed by comparison of the enzymes isolated by an improved procedure (Spindler et al., 1978).

To examine the mechanism responsible for the regulation of rRNA transcription, we are developing an in vitro system of purified and characterized components. Utilizing bacteriophage λ as a vector, we have constructed recombinants containing *Eco*RI framents of the entire *Acanthamoeba* nuclear genome. One of these, λCh9Ar1, was derived from a partial *Eco*RI digest and contains one complete rDNA repeat. This recombinant phage has the topological structure: 5′-λ left arm-phage internal fragment-5.8 S-26 S-nontranscribed spacer-18 S-λ right arm-3′. The likelihood that the 18S rDNA is at the 5′ end of the initial transcript (Long & Dawid, 1980) coupled with our finding that the sense strand of the rDNA insert is the r strand (rightwardly transcribed) of the recombinant phage suggests that the promotor region lies near the 3′ end of the nontranscribed spacer. This region has been subcloned into pBR322, and the resulting plasmid (pAr1) is an excellent template for in vitro transcription studies.

Experimental Procedures

Bacteria and Phage. *Escherichia coli* C600 (*thr leu tonA lacY thi hsr hsm*) was the bacterial host used for the propagation of phage. NS428, NS204 (*Aam*11 *b2 red*3 c*I*857 *Sam*7) and λ*dg*805 (λ*dga*1805 c*I*857 *Sam*7) were the lysogenized strains used to prepare packaging extracts and phage A protein (Becker & Gold, 1975). The EK1 certified phage Charon 9 was utilized as the vector for shotgun cloning of the *Acanthamoeba* nuclear genome. *E. coli* SK1592 (*gal thi ton sbcB*15 *endA hsr*; Kushner, 1978) was the host for subcloning of plasmids constructed between pBR322 and recombinant phage DNA fragments.

Topological Mapping of Cloned Fragments. Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and were used as recommended by the supplier. When multiple restrictions were performed, each individual reaction was terminated by heating to 65 °C for 5 min prior to the addition of the subsequent enzyme. Restriction fragments were separated in 0.7% (w/v) agarose slab gels in 40 mM Tris–acetate, pH 8.1, 5 mM sodium acetate, and 1 mM EDTA at potential of 5 V/cm. Smaller fragments [<2 kbp (kilobase pairs)] were resolved in 5% polyacrylamide slab gels containing 3.3% (w/w) *N,N′*-methylenebis(acrylamide) at a potential of 10 V/cm by using the same buffer as described above. Gels were stained for 30 min in ethidium bromide (0.5 μg/mL in water), illuminated with shortwave UV light, and photographed with Kodak Tri-X panchromatic film. The coding regions of cloned ribosomal DNA fragments were mapped as described by Southern (1976) with BA85 nitrocellulose filters (Schleicher & Schuell, Keene, NH). The processed filters were hybridized with 2.5×10^5 dpm of 5.8S, 18S, or 26S ^{125}I-labeled rRNA (specific activity $\sim 5 \times 10^6$ dpm/μg; Commerford, 1976). Hybridization was at 37 °C for 12 h in 50% formamide and 5× SSCP (1× SSCP is 50 mM KH_2PO_4, 15 mM trisodium citrate, 130 mM NaCl, and 1 mM EDTA, pH 7.2; Benton & Davis, 1977). The filters were washed for 1 h in the hydridization buffer at 37 °C followed by one wash in 5× SSCP and one wash in 2× SSCP (15 min each). The filters were exposed to Kodak XR-5 X-ray film at −80 °C for 12–24 h with Du Pont Lightning Plus intensification screens.

Cloning of Acanthamoeba Nuclear DNA in the Charon Phages. Charon 9 will accept inserts of 10.2–24.4 kbp (Blattner et al., 1977). In order to exploit the large capacity of this phage, we produced partial *Eco*RI digests of *Acanthamoeba* nuclear DNA for cloning in this vector. This was done by empirically determining reaction conditions for *Eco*RI digestion which yielded fragments of 16-kbp average size. The relative concentrations of restricted phage DNA and *Eco*RI *Acanthamoeba* DNA fragments, as well as the total concen-

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tration of DNA in the ligation mix, were calculated as described (Blattner et al., 1977) by assuming an average size of 16 kbp for the fragments produced by partial *Eco*RI digestion. Ligation was performed in 20 μL containing 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 1 mM ATP (sodium salt), 10 mM dithiothreitol, and 1.4 units of T4 DNA ligase (New England Biolabs, Beverly, MA) for 24 h at 16 °C. A 4-μL sample of ligation mix containing 1 μg or less of DNA was used in each packaging reaction (200 μL); all packaging extracts and other components were prepared and used as described in the detailed protocols which accompany the Charon phage kit obtained from Dr. Frederick Blattner, except that the stage I reaction was performed for 15 min at 22 °C and the stage II reaction for 1 h at 35 °C (Sternberg et al., 1977). These modifications were found to increase the efficiency of packaging (the ratio of plaques out to phage genomes in) severalfold. Under these conditions, we determined the efficiency of packaging to be 1×10^{-6} for the Charon 9/*Acanthamoeba Eco*RI partials. The phages from the in vitro packaging reaction were plated at 500–1000 plaques per 10 cm diameter dish and screened as described by Benton & Davis (1977) with 18S and 26S ^{125}I-labeled rRNA. Filters were exposed as described for Southern transfers and positive signals confirmed by plaque purification and rescreening.

Growth of Phage and Purification from Large-Scale Lysates. Primary phage stocks were produced from single plaques by impaling the plaque with a Pasteur pipet and allowing it to soak for 30 min in PSB buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, and 100 μg/mL gelatin). A 100-μL sample was mixed with 100 μL of stationary phase C600 and 100 μL of Mg–Ca salts (10 mM $MgCl_2$ and 10 mM $CaCl_2$); this was allowed to adsorb for 10 min at 37 °C and used to inoculate 50 mL of NZYC broth [10 g of NZamine (Humko-Scheffield, Lynhurst, NJ), 5 g of yeast extract, 1 g of casamino acids (both Difco, Detroit, MI), 5 g of NaCl, and 2 g of $MgCl_2 \cdot 7H_2O$ per L]. For the growth of large-scale lysates, 5×10^5 phages from a titered primary in 0.3 mL of PSB were mixed with 0.3 mL of stationary phase C600 and 0.3 mL of Mg–Ca salts. After preadsorption as above, the mixture was used to inoculate 1 L of NZYC broth. Lysis generally ensued 8–12 h after vigorous aeration at 38 °C was begun, and lysates routinely yielded $(1–2) \times 10^{10}$ phages/mL. The most significant variable affecting phage titer was the bacterial strain used for growth. We have had consistently poor growth of both parental and recombinant phages in CSH18 and K802 which could not be circumvented by altering the multiplicity of infection and/or the pH of the broth.

Phages were purified from the cleared lysates as described (Blattner et al., 1977) except that the poly(ethylene glycol) precipitation (PEG 6000; Sigma Chemicals, St. Louis, MO) was for 1 h at 4 °C in 0.5 M NaCl with 10% (w/v) PEG (Yamamoto & Alberts, 1970). The phage band obtained from the second CsCl centrifugation was dialyzed against 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM $MgCl_2$ and stored in the cold.

Preparation of Phage DNA. DNA was prepared from 1×10^{13} phages in 1 mL, assuming that an absorbance of unity at 260 nm (1-cm path length) corresponds to 8×10^{11} phages/mL (Szybalski et al., 1971). The phages were disrupted by the addition of EDTA to 25 mM and sodium dodecyl sulfate to 0.5% followed by incubation at 65 °C for 15 min. Proteinase K (EM Laboratories, Darmstadt) was added to 0.5 mg/mL and the lysate incubated for 2 h at 37 °C. The DNA was extracted twice with phenol (Mallinckrodt 0028, used directly after saturation with 0.1 M Tris-HCl, pH 7.4, and 10 mM EDTA) followed by two extractions with chloroform–isoamyl alcohol (24:1 v/v). The DNA was exhaustively dialyzed against 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA and the concentration determined by absorbance at 260 nm.

Strand Separation of Cloned DNA. The l and r strands of the recombinant phage were separated by isopycnic banding in CsCl gradients by the poly[ribo(U,G)] method of Szybalski et al. (1971). The strand-separated DNAs were subjected to alkaline hydrolysis in 0.1 N NaOH for 14 h at 37 °C followed by neutralization, self-annealing in 6× SSC (1× SSC is 0.015 M trisodium citrate, pH 7.0, and 0.15 M NaCl) for 2 h at 65 °C. The DNA was loaded onto nitrocellulose filters (Bovre & Szybalski, 1971) and hybridized with mixture of 18S and 26S ^{125}I-labeled rRNA for 18 h by using the Benton–Davis hydridization protocol and washing procedure (Benton & Davis, 1977). The dried filters were counted in a Packard Tri-Carb liquid scintillation spectrometer with a toluene-based cocktail.

Subcloning of λChAr1 Genomic Fragments. Clones containing eukaryotic fragments of λCh9Ar1 were constructed by standard techniques. The vector pBR322 was *Eco*RI digested, treated with bacterial alkaline phosphatase, and ligated to the gel-purified 5.1-kbp *Eco*RI fragment of λCh9Ar1 which contains the 26S and 5.8S rRNA genes. The recombinant molecules were used to transform SK1592 (Dagert & Erhlich, 1979) and colonies screened with ^{125}I-labeled 26S rRNA according to Grunstein & Hogness (1975). The 6.9-kbp *Eco*RI *Acanthamoeba* fragment of λCh9Ar1, which contains most of the 18S rRNA gene, resisted similar attempts to be cloned into pBR322. Therefore, the slightly shorter 6.5-kbp *Bam*HI–*Eco*RI fragment was cloned into pBR322 digestion with these enzymes (this fragment contains the identical 18S rRNA coding region as the parental fragments). The 0.35-kbp *Eco*RI–*Bam*HI fragment of pBR322 was removed by sucrose-gradient centrifugation and the 4.0-kbp *Eco*RI–*Bam*HI fragment used as the vector. After ligation of the vector and the gel-purified 6.5-kbp *Acanthamoeba* fragment, *E. coli* SK1592 was transformed with the chimeric DNA. Tetracycline-sensitive and ampicillin-resistant colonies were screened with ^{125}I-labeled 18S rRNA to find the clones in interest.

A colony containing the 6.5-kbp *Eco*RI–*Bam*HI fragment was designated pAr1, while a colony containing the 5.1-kbp *Eco*RI fragment was designated pAr2.

R-Loop Methods. The R-loop hybridization solution (Thomas et al., 1976) contained 70% formamide (deionized with Amberlite mixed bed), 0.08 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer at pH 7.9, 0.08 M sodium acetate, 10 mM EDTA, 4–6 μg of purified λCh9Ar1 DNA, and 4–6 μg of sucrose gradient purified *Acanthamoeba* 18S or 26S rRNA. The final volume was 60 μL. The solutions were sealed in 100-μL siliconized glass capillary tubes and incubated at 49 °C for 6–7 h.

Aliquots of the hybridization solutions were prepared for electron microscopy by using the formamide variation of the basic protein film technique (Westmoreland et al., 1969; Davis et al., 1971). The hyperphase was 0.1 M Tris-HCl, pH 8.1, 10 mM EDTA, and 47% formamide with 0.1 μg/mL RF II φX174 as an internal standard in a final volume of 100 μL. To this was added 2 μL of the hydridization solution followed by 5 μL of 1 mg/mL cytochrome *c*. The entire hyperphase was immediately spread across a hypophase (0.01 M Tris-HCl, pH 8.4, 10 mM EDTA, and 17% formamide) in a 15-cm^2 plastic petri dish. Approximately 45 s after being spread, the

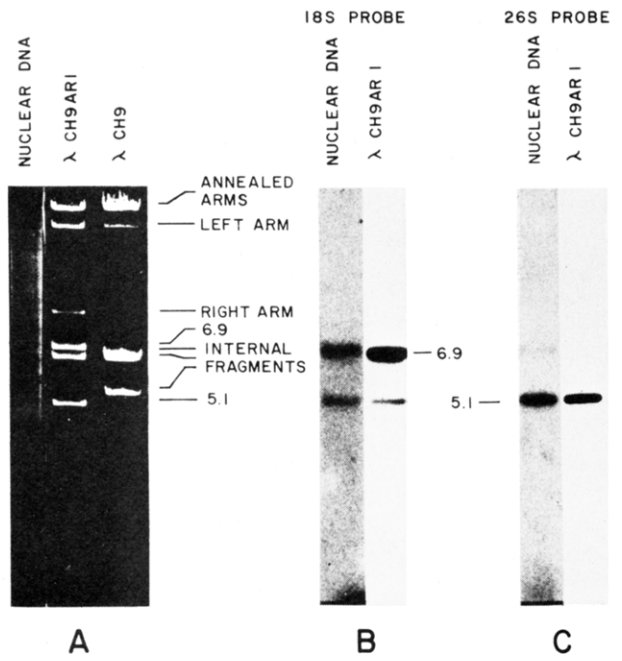

FIGURE 1: Hybridization of ^{125}I-labeled 18S or 26S rRNA to Southern blots of genomic or cloned DNA *Eco*RI digests. (A) A 1-μg sample of *Acanthamoeba* nuclear DNA or Ch9Ar1 DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Duplicate Southern transfers were challenged with 18S (B) or 26S (C) iodinated rRNA and hybridized fragments detected by autoradiography. An *Eco*RI digest of λCh9 DNA is shown in (A) for comparison; sizes are given in kilobase pairs (kbp).

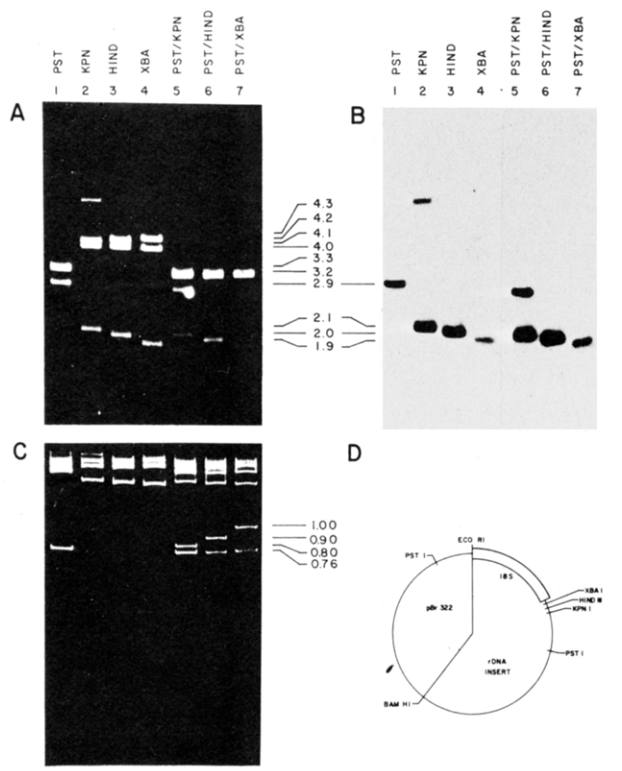

FIGURE 2: Topological map of pAr1. Aliquots of pAr1 DNA were digested with various restriction enzymes and separated by electrophoresis on (A) 0.7% agarose or (C) 5% acrylamide. (B) A Southern transfer of the agarose gel was hybridized with ^{125}I-labeled 18S rRNA and autoradiographed. (D) A map constructed from the data of (A–C). All sizes are in kbp.

protein film was picked up on collodion-coated 300-mesh copper grids. The samples were stained with 5×10^{-5} M uranyl acetate in 90% ethanol, destained with 90% ethanol, and rotary shadowed with platinum–palladium.

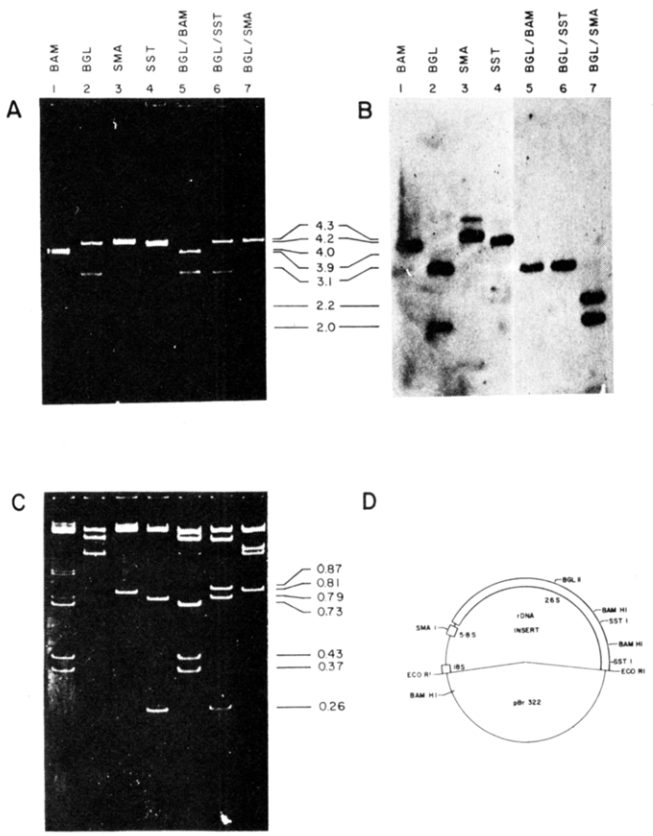

FIGURE 3: Topological map of pAr2. Aliquots of pAr2 DNA were digested with restriction enzymes and separated by electrophoresis on (A) 0.7% agarose or (C) 5% acrylamide. (B) A Southern transfer of the agarose gel hybridized with ^{125}I-labeled 26S rRNA and autoradiographed. (D) A map constructed from the data of (A–C). All sizes are in kbp.

Results

In order to establish a cloning strategy, nuclear DNA from *Acanthamoeba* was digested with *Eco*RI, and the resulting fragments were separated by agarose gel electrophoresis and analyzed by the Southern technique. Two DNA fragments of 5.1 and 6.9 kbp which hybridized to 26S and 18S rRNA, respectively, were obtained (Figure 1). A similar experiment with *Pst*I produces a single 12-kbp fragment which hybridizes with both probes, strongly suggesting that the two *Eco*RI fragments comprise the entire ribosomal DNA repeat (data not shown). In order to construct a recombinant DNA containing the rDNA transcription unit in its entirety, a partial *Eco*RI digest of *Acanthamoeba* nuclear DNA was ligated to *Eco*RI-digested λCh9. The recombinant DNAs were packaged in vitro and used to transfect *E. coli* CSH 18. Approximately 6000 plaques were screened (Benton & Davis, 1977), and three of the positive recombinant phages were shown to contain both 18S and 26S rDNA sequences (for example, see Figure 1). One of these, λCh9Ar1, was selected for more detailed mapping.

For facilitation of restriction enzyme mapping, fragments of λCh9Ar1 were subcloned into the vector pBR322 and mapped; with this information, determination of the manner in which the fragments were oriented in the recombinant phage was simplified (Figures 2 and 3). (Confer, also, Figures 4 and 5; see paragraph at end of paper regarding supplementary material.) The final map is shown in Figure 6.

26S rRNA Is Encoded by Two Noncontiguous Regions. For confirmation of the location of the coding sequences within

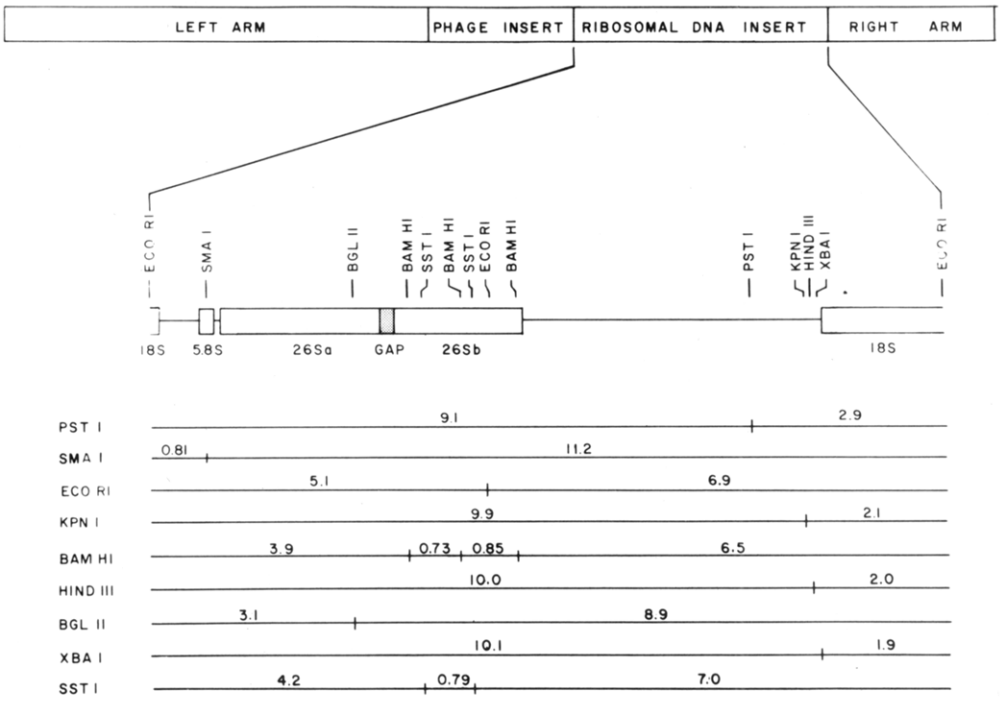

FIGURE 6: Topological map of λCh9Ar1. The orientation of the cloned insert within the phage, the order of the restriction sites, and the fragment sizes generated by nine restriction enzymes were determined as described under Experimental Procedures and Supplementary Material. All sizes are in kbp.

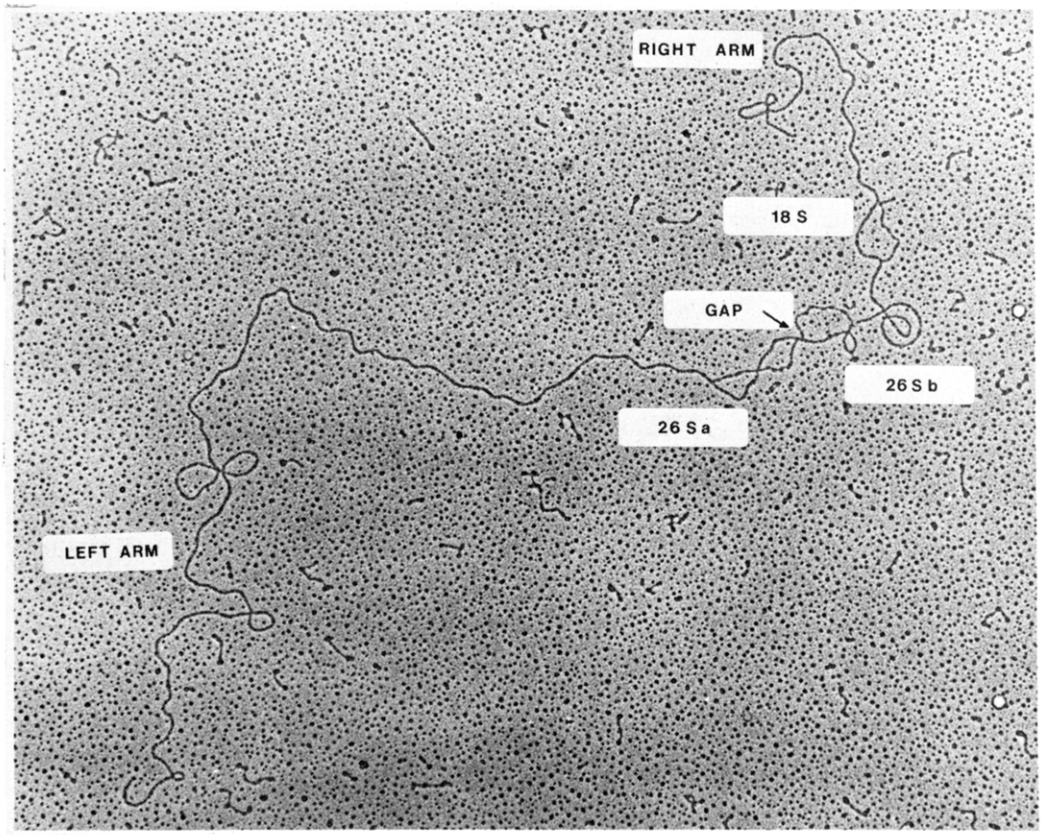

FIGURE 7: R loops formed between λCh9Ar1 DNA and *Acanthamoeba* rRNA. R loops were formed as described under Experimental Procedures and spread for electron microscopy.

λCh9Ar1, R loops were formed between 18S and 26S rRNA and the recombinant phage DNA and prepared for electron microscopy (Figure 7 and Table I). In addition to confirming the map coordinates of the coding sequences, other information was derived from the R loops. Stevens & Pachler (1972) demonstrated that the 26S rRNA of *Acanthamoeba* is made up of two large RNA fragments (in addition to the associated 5.8S rRNA). We have estimated these to be 2000 and 2400 nucleotides in length by electrophoresis of the glyoxalated RNAs in agarose gels (J. M. D'Alessio, unpublished experiments). The R-loop data reveal that the coding sequence for the larger of these is proximal to the 5.8S rRNA gene, and the coding sequences for these two large RNAs are separated by a gap of approximately 200 base pairs. Presumably, this

Table I: Comparison of Map Coordinates of the Ribosomal RNA Genes of λCh9Ar1 from R-Loop Mapping and Gel Electrophoretic Analysis

description	length (kbp)	
	R loops[a]	gel analyses
length of λCh9Ar1	50.1 ± 2.48	47.7
end of left arm to 26S gene	29.6 ± 1.68	27.7
end of right arm to 18S gene	9.32 ± 0.53	8.80

[a] Average of duplicate measurements of five molecules ± standard deviation with φX174 RFII DNA as a standard (5.39 kbp).

material is removed during processing of the precursor. Furthermore, no introns are apparent within the R loops formed to the coding sequences of either the 18S or the 26S genes.

λCh9Ar1 Contains a Permuted Arrangement of the Ribosomal RNA Transcription Unit. In those eukaryotes studied to date, the precursor rRNA transcript has its 18S sequence proximal to the 5′ terminus (Perry, 1976). In addition, the 5.8S rRNA has been found to be bracketed by the 18S and 26S rRNAs. If this is also the case in *Acanthamoeba*, then the cloned DNA fragment in *λCh9Ar1* must have arisen by cleavage at homologous *Eco*RI sites in two successive transcription units which are arranged head-to-tail in the genome. This interpretation is supported by a number of findings: (1) the arrangement of the 18S, 5.8S, and 26S rRNA coding sequences seen in the recombinant is permuted relative to the expected order of appearance in the transcription unit; (2) if all of the coding sequences plus the large noncoding sequence surrounding the unique *Pst*I site were transcribed, a precursor RNA of 12 kilobases would result. Since the putative precursor in *Acanthamoeba* is 37 S (approximately 8700 nucleotides; Stevens & Pachler, 1972; our own observations), 2–3 kbp of the cloned unit are not transcribed. (3) When the separated stands of λCh9Ar1 are hybridized to ^{125}I-labeled 18S + 26S rRNA, only the r strand hybridized (r = 90 000 cpm, l = 500 cpm). The cloned DNA fragment must be transcribed in the rightward direction, assuming that the strands of λCh9Ar1 separate in the same manner as wild-type λ, and (4) sequences to the left of the single *Xba*I site of the 6.5-kbp insert of pAr1 are not transcribed. This was demonstrated by hybridization of in vitro 5′-^{32}P-labeled 37S rRNA to Southern blots of the pAr1 insert digested with *Xba*I, *Hin*dIII, *Kpn*I, or *Pst*I (Figure 8). We conclude that the 37S precursor initiates close to the *Xba*I site and proceeds rightward through the 18S, 5.8S, and 26S genes.

5S rRNA Is Unlinked to the Large Ribosomal Transcription Unit. In bacterial cells, in eukaryotic organelles, and in some lower eukaryotic nuclear genomes, the 5S rDNA is linked to the large ribosomal RNA transcription unit (Attardi & Amaldi, 1970; Long & Dawid, 1980). Radiolabeled 5S rRNA from *Acanthamoeba* does not hybridize with λCh9Ar1 nor does it hybridize with the *Eco*RI fragments of the transcription unit when these are present in a digest of nuclear *Acanthamoeba* DNA. Thus, the 5S RNA gene is not linked to the ribosomal RNA repeat unit. Therefore, *Acanthamoeba* may be on the other side of an evolutionary juncture distinguishing bacteria and lower eukaryotes from higher organisms.

Discussion

Southern blots to restriction endonuclease digested *Acanthamoeba* nuclear DNA show that the rRNA transcription unit is 12 kbp in length. The entire transcription unit has been cloned in λCh9, and fragments have been subcloned into pBR322. These clones have been mapped with ten restriction enzymes. The coding regions for 18S and 26S rRNAs were identified by hydridization of radiolabeled rRNA to Southern blots and by R-loop mapping. The map is shown in Figure 6.

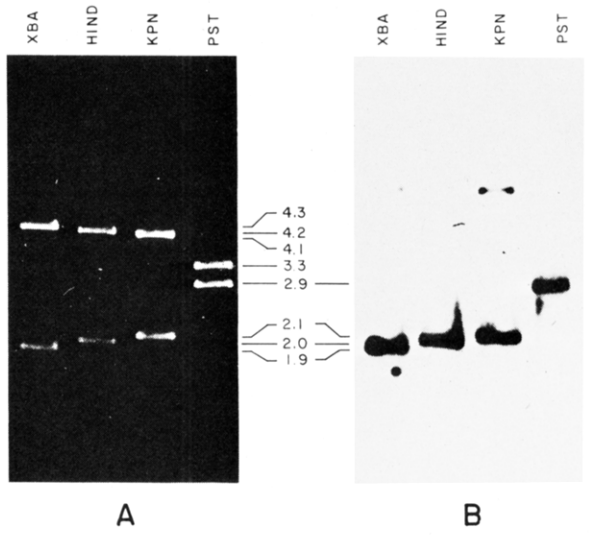

FIGURE 8: Initiation sequence for 37S preribosomal RNA lies in the vicinity of the Xba*I* site of pAr1. (A) The 6.5-kbp insert of pAr1 was digested with various restriction enzymes and separated by electrophoresis on a 0.7% agarose gel. (B) A Southern blot of the fragments was challenged with 2.5 × 10^5 dpm of 37S preribosomal RNA [isolated from [^{3}H]uridine pulse-labeled cells by sucrose-gradient centrifugation of nuclear RNAs (Stevens & Pachler, 1972)] labeled at the 5′ terminus with ^{32}P by using T4 polynucleotide kinase and [γ-^{32}P]ATP. Fragment sizes are in kbp.

The size of the ribosomal RNA repeat unit in eukaryotic cells is quite variable from species to species [cf. 9 kbp in yeast (Bell et al., 1977), 40 kbp in man and mouse (Arnheim & Southern, 1977), 28 kbp in *Physarum* (Vogt & Braun, 1976; Molgaard et al., 1976)]. This variation resides mainly in the nontranscribed spacer (NTS) region. The transcribed region varies only between 8 and 13 kbp. Not only is there large variability in the size of the NTS between species, but, in several species, heterogeneity exists in the NTS within a given cell (Long & Dawid, 1980). The *Acanthamoeba* repeat is 12 kbp, near the lower end of the size range. Only one repeat unit type was observed in Southern blots of *Acanthamoeba* DNA, and all *Eco*RI clones of the ribosomal DNA contained fragments of identical sizes. Therefore, there does not appear to be significant size heterogeneity in the NTS nor in the coding regions of *Acanthamoeba* rDNA.

The cloned transcription unit has a permuted arrangement of coding regions. This arrangement is consistent with several possibilities: (1) arrays of tandemly repeated genes, grouped in a head-to-tail fashion and integrated into the genome, (2) two or more repeats making up an extrachromosomal covalently closed circular molecule, or (3) very large linear extrachromosomal DNAs containing many repeats. Initial experiments designed to differentiate among these possibilities suggest that the ribosomal RNA genes are located on DNA fragments of discreet sizes which, if linear, are multiples of an approximately 60-kbp unit (J. M. D'Alessio, unpublished experiments). The number of 12-kbp repeat units per 60-kbp molecule is not known nor has it been determined if the DNAs are palindromic.

The repeat unit codes for a precursor RNA which is processed into 18S, 5.8S, 26Sa, and 26Sb RNAs. Several lines of evidence presented here support the notion that, as in all other eukaryotic and bacterial rRNA repeat units, the polarity

of transcription proceeds 5′-18 S-5.8 S-26 S-3′. The 26Sa, 26Sb, and 5.8S RNAs are found as a single base paired complex in the large ribosomal subunit (L. Thiers and T. Pitman, unpublished experiments; Stevens & Pachler, 1972), and the coding regions for 26Sa (2400 base pairs) and 26Sb (2000 base pairs) are separated by a gap of approximately 200 base pairs. 5S RNA is also found in the large ribosomal subunit. However, unlike some lower eukaryotic cells (Bell et al., 1977; Maizels, 1976), the 5S RNA gene is not physically linked to the repeat unit.

Hybridization of the labeled putative 37S preribosomal RNA to Southern blots of appropriate fragments of cloned DNA places the 5′ end of the precursor close to the unique *Xba*I site within the repeat. Therefore, the subclone of a fragment containing this region (pAr1) is an excellent template for both in vitro transcription studies and more detailed structural studies. We are currently developing an in vitro transcription system utilizing the purified and characterized RNA polymerase I from *Acanthamoeba* and this DNA. Detailed mapping of the initiation site for the 37S precursor is also in progress.

Acknowledgments

These experiments were carried out in accordance with NIH Guidelines for Recombinant DNA Research and were performed under P2/EK1 containment. We gratefully acknowledge the assistance of Dr. Thomas Cech in the preparation of R-loops for electron microscopy.

Supplementary Material Available

A detailed explanation of the derivation of the pAr1 and pAr2 restriction site topology and an explanation of λCh9Ar1 restriction fragment ordering (7 pages). Ordering information is given on any current masthead page.

References

Arnheim, N., & Southern, E. M. (1977) *Cell (Cambridge, Mass.) 11*, 363–370.

Attardi, G., & Amaldi, F. (1970) *Annu. Rev. Biochem. 39*, 183–226.

Becker, A., & Gold, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 581–585.

Bell, G. I., DeGennaro, L. J., Gelfand, D. H., Bishop, R. J., Valenzuela, P., & Rutter, W. J. (1977) *J. Biol. Chem. 252*, 8118–8125.

Benton, W. D., & Davis, R. W. (1977) *Science (Washington, D.C.) 196*, 180–182.

Blattner, F. R., Williams, B. G., Blechl, A. E., Denniston-Thompson, K., Faber, H. E., Furlong, L., Grunwald, D. J., Kiefer, D. O., Moore, D. D., Schumm, J. W., Sheldon, E. L., & Smithies, O. (1977) *Science (Washington, D.C.) 196*, 161–169.

Bovre, K., & Szybalski, W. (1971) *Methods Enzymol. 21D*, 350–383.

Commerford, S. L. (1971) *Biochemistry 10*, 1993–2000.

Dagert, M., & Erhlich, S. D. (1979) *Gene 6*, 23–28.

Davis, R. W., Simon, M., & Davidson, N. (1971) *Methods Enzymol. 21D*, 413–428.

Detke, S., & Paule, M. R. (1975) *Biochim. Biophys. Acta 383*, 67–77.

Detke, S., & Paule, M. R. (1976) *ICN-UCLA Symp. Mol. Cell. Biol. 5*, 261–266.

Grunstein, M., & Hogness, D. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3961–3965.

Kushner, S. R. (1978) in *Genetic Engineering* (Boyer, H. B., & Nicosia, S., Eds.) pp 17–23, Elsevier, Amsterdam.

Long, E. O., & Dawid, I. B. (1980) *Annu. Rev. Biochem. 49*, 727–764.

Maizels, N. (1976) *Cell (Cambridge, Mass.) 9*, 431–438.

Molgaard, H. V., Mathews, H. R., & Bradbury, E. M. (1976) *Eur. J. Biochem. 68*, 541–549.

Perry, R. P. (1976) *Annu. Rev. Biochem. 45*, 605–629.

Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.

Spindler, S. R., Duester, G. L., D'Alessio, J. M., & Paule, M. R. (1978) *J. Biol. Chem. 253*, 4669–4675.

Sternberg, N., Tiemeier, D., & Enquist, L. (1977) *Gene 1*, 255–280.

Stevens, A. R., & Pachler, P. F. (1972) *J. Mol. Biol. 66*, 225–237.

Stevens, A. R., & Pachler, P. F. (1973) *J. Cell Biol. 57*, 525–537.

Szybalski, W., Kubinski, H., Hradecna, Z., & Summers, W. C. (1971) *Methods Enzymol. 21D*, 333–413.

Thomas, M., White, R. L., & Davis, R. W. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2294–2298.

Vogt, V. M., & Braun, R. (1976) *J. Mol. Biol. 106*, 567–587.

Westmoreland, B. C., Szybalski, W., & Ris, H. (1969) *Science (Washington, D.C.) 163*, 1343–1348.

Yamamoto, K. R., & Alberts, B. M. (1970) *Virology 40*, 734–744.