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Unique sequence arrangement of ribosomal genes in the palindromic rDNA molecule of Physarum polycephalum

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ABSTRACT

R-loop and restriction mapping procedures reveal the organization of coding regions at each end of the giant rDNA palindrome of Physarum polycephalum. A 19S coding region of 2.10 ± 0.21 kb is located at each end of a very long central spacer (35.64 ± 2.08 kb). An internal spacer of 1.66 ± 0.12 kb lies distal to the 19S gene. The 5.8S rRNA coding region is located in this spacer. The 26S gene lies distal to the internal spacer. The 26S gene is unusual among those of eukaryotes in that it consists of 3 coding regions (α, β and γ) interrupted by 2 intervening sequences. The 26S α (most central) coding segment of 2.41 ± 0.33 kb is separated from the 26S β segment by an intervening sequence of 0.68 ± 0.13 kb. The 26S β segment (0.70 ± 0.11 kb) is separated from the most distal 26S γ segment (0.59 ± 0.14 kb) by an intervening sequence of 1.21 ± 0.14 kb. The 2 intervening sequences are present in at least 88% of ribosomal genes from active plasmodia, indicating that genes containing these sequences are transcribed. The rDNA termini contain a heterogeneous region which varies in length by ± 300 base pairs.

INTRODUCTION

Ribosomal RNA of Physarum polycephalum is encoded on multiple copies of an extrachromosomal nucleolar satellite DNA molecule (1-7). Each rDNA molecule exists as a giant palindromic sequence, as demonstrated by electron microscopy of denatured rDNA fold-back structures (8) and by cleavage with restriction enzymes Hind III and Eco RI (8,9). Since milligram quantities of rDNA can be isolated in nucleoli, together with associated histones and DNA-binding proteins, Physarum offers unique advantages as a system for studies involving ribosomal gene packaging and regulation. Most of these studies depend upon accurate mapping of the rDNA molecule. It is known that the rDNA carries genes coding for 5.8S, 19S, and 26S rRNA species, but not for 5S or tRNAs (6). Studies of Molgaard et al. (9) have demonstrated the palindromic arrangement of 19S and 26S genes. However, at this time little is known regarding the precise coordinates and internal sequence organization of the rRNA coding regions. In this study we have employed R-loop and restriction mapping procedures to deter-

mine the positions of the 5.8S, 19S and 26S ribosomal genes. Our results reveal an unusual configuration of 26S coding sequences and localize a source of length heterogeneity at the rDNA termini.

METHODS

Preparation of rDNA. Nuclei of Physarum (strain a x i) microplasmodia, grown in 3 to 5 5L flasks (10) were isolated as previously described (3, 11). Nuclei were then disrupted by passage through a French pressure cell at 8000 psi (12), and nucleoli prepared essentially as described by Mohberg and Rusch (11). DNA was extracted from nucleoli by the method of Gross-Bellard et al. (13), and rDNA was purified by CsCl density gradient centrifugation (3). Purity of rDNA, which has a characteristic buoyant density of 1.713 g/cc in CsCl (3), was monitored by analytical ultracentrifugation using a Beckman UV Prep Analyzer.

R-loop mapping. R-loop hybridization (14) was performed for 4 hrs at 45° in a solution containing 80% formamide, 0.3M NaCl, 10mM EDTA, 50mM TES buffer, pH 7.0, 10 μg/ml of purified rDNA and 10 μg/ml of 19S or 26S rRNA. Samples were diluted 50 to 200-fold into buffer containing 50% formamide, 10mM EDTA and 0.1M Tris-HCl, pH 8.5, for spreading. Following addition of cytochrome c to 50 μg/ml, samples were spread on a hypophase of 20% formamide, 10mM Tris-HCl, pH 8.5. Grids were stained with uranyl acetate and rotary shadowed with Pt-Pd (15). Length measurements were made by inclusion of φx174 single stranded or replicative form DNA in hybridization mixtures. The length of the φx174 genome was taken to be 5386 nucleotides (16). In agreement with Philippsen et al. (17), we find that RNA/DNA duplexes are approximately 4% shorter than corresponding DNA/DNA duplexes. Spreading from 70% formamide did not appreciably influence the lengths of R-loops measured.

Restriction endonuclease cleavage. Purified rDNA was incubated with various restriction endonucleases (obtained from New England Biolabs) according to standard procedures (c.f. ref. 18). DNA samples were subjected to electrophoresis in 1.0% or 1.4% agarose gels using the procedure of Murray and Murray (18) with minor modifications. DNA bands were visualized under UV light following staining with ethidium bromide (1.0 μg/ml) for 30 min at room temperature. DNA fragments were transferred from gels to nitrocellulose filters using the procedure of Southern (19).

Preparation of ^{32}P-labeled rRNA species. 19S and 26S RNAs were prepared as described (12, 20) from macroplasmodia labeled in vivo with 5 mCi of ^{32}P-phosphate (New England Nuclear; carrier-free as NaH_2PO_4 in water) per 2 x 10^8

nuclei for 72 hrs. Specific activities of rRNA were 5-7 x 10^5 cpm/μg.

5.8S rRNA was purified from the 26S fraction of an rRNA preparation from a culture which had been labeled with ^{3}H-uridine. After heating for 2 min at 69°, 26S rRNA, dissolved in 1.0mM EDTA, 0.5% SDS, 0.1M NaCl, 20% formamide and 5.0mM Tris-HCl, pH 7.4, was cooled to room temperature and recentrifuged through a 15%-30% sucrose gradient. All RNA smaller than 10S was pooled, and, after precipitation from cold ethanol, subjected to electrophoresis for 2.5 hrs throuth a cylindrical 5% polyacrylamide gel (20). The gel was scanned at 265nm, and the RNA from the only detectable peak was eluted by incubating the gel section in 0.1M NaCl at 30° for 18 hrs. Purity of the eluted 5.8S rRNA was monitored by electrophoresis of an aliquot in the presence of added nonradioactive 4S and 5S markers in a 5% polyacrylamide gel. The remainder of the eluted 5.8S RNA was precipitated from 73% ethanol at -30°.

The 5.8S RNA was labeled with ^{32}P using T4 polynucleotide kinase (P.-L. Biochemicals) with γ-^{32}P-ATP as a substrate (New England Nuclear; 10 Ci/mmole), following limited alkaline hydrolysis of RNA, according to the procedure of Maizels (21). Incorporation of radioactive phosphate into 5.8S RNA was 6 to 9 x 10^7 cpm/μg.

Hybridization. Hybridization was carried out in 49% formamide, 0.9M NaCl, 0.1% SDS, 1.0mM EDTA, and 25mM TES buffer, pH 7.0, using 1-2 x 10^5 cpm of labeled RNA per 1 cm filter strip, or 1-2 x 10^6 cpm for a full 15 cm x 15 cm filter. Filters wet with hybridization buffer were wrapped in Saran Wrap and incubated for 18 hrs at 50°. After incubation, filters were washed once with 50 ml of hybridization buffer at 50°, and rinsed 3 times with 2x SSC. Filters were then treated with ribonucleases A (Sigma; 10μg/ml) and T1 (Worthington; 5U/ml) for 1 hr at room temperature in 50 ml of 2x SSC, followed by 3 more rinses with 2x SSC. Filters were then dried under vacuum at 80° and autoradiographed using DuPont Cronex X-ray film.

RESULTS

R-loop mapping of Physarum ribosomal genes. Purified rDNA was hybridized with 19S and 26S rRNA species to form R-loops, loops resulting from displacement of a single DNA strand from a DNA duplex by complementary RNA (14). Fig 1 is an electron micrograph of a typical intact rDNA molecule possessing 19S and 26S gene R-loops at each end. These results are the first direct visualization of a palindromic arrangement of rRNA coding regions. All DNA strands unambiguously identified as intact rDNA molecules were observed as linear palin-

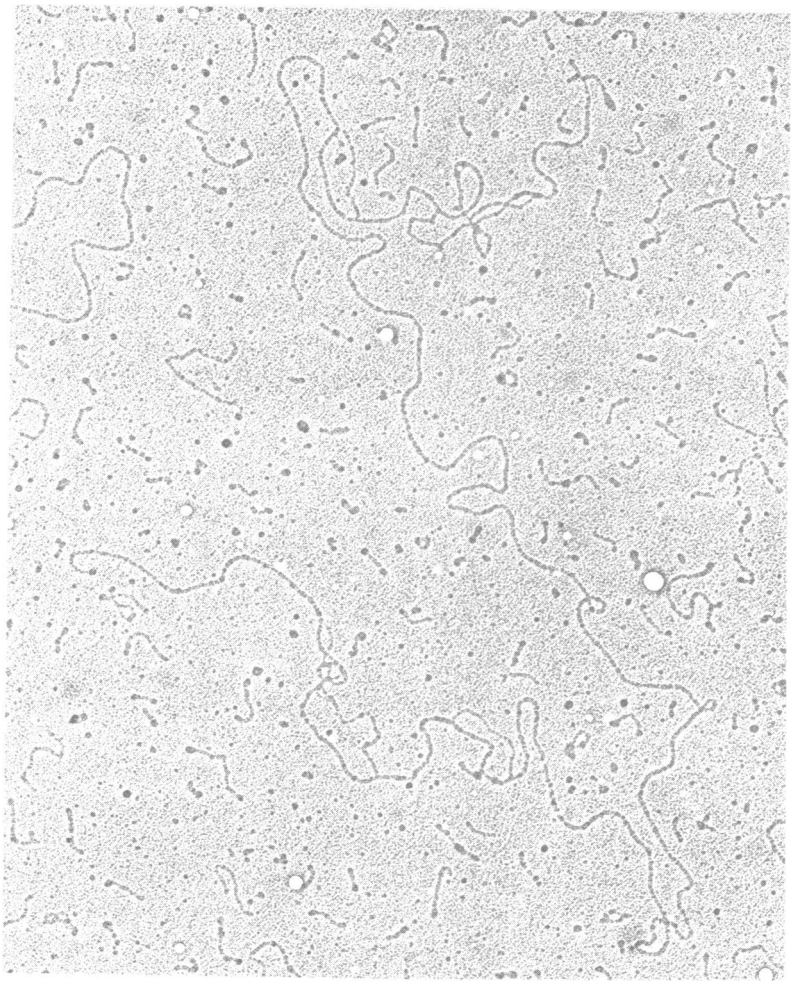


Fig 1a. 19S and 26S R-loops formed on a single, intact rDNA palindrome from Physarum microplasmodia. Hybridization and electron microscopy were conducted as described in the text. Magnification x 39,000. Graphic interpretation on adjacent page (Fig 1b).

dromes of approximately 61 kb (39 Mdal). No circular molecules or tandem arrays of genes were seen. Only about 6% variation could be detected in the length of the central spacer (that segment separating the 19S genes). The average length of this spacer is 35.64 ± 2.08 kb. Lengths of coding sequences were calculated by comparing lengths of RNA/DNA duplex portions of loops with

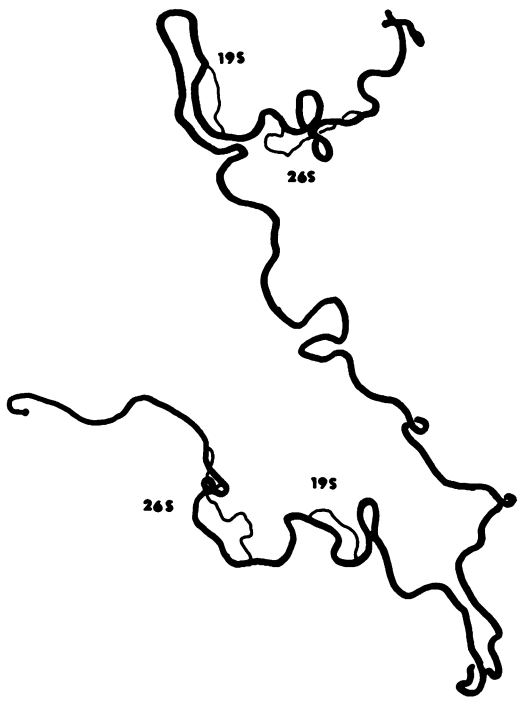


Fig 1b. Graphic representation of the intact rDNA palindrome shown in Fig 1a. Note the complexity of 26S gene loop structures and secondary structure at the rDNA termini.

lengths of ϕx174 DNA included as internal standards, as described in Methods. The 19S gene was always visualized as a single R-loop. The average length of the 19S rRNA coding region was calculated to be 2.10± 0.21 kb. Variation in length measurements of R-loops could be due to branch migration or to reannealing of DNA and displacement of RNA at loop forks. However, spreading from high formamide concentrations, reported to hinder such reannealing (17) had little effect on length measurements in the present study.

As seen in Fig 1, the 26S R-loop was frequently seen as a complex structure in which the 2 sides of the loop were difficult to distinguish. However, the majority of 26S R-loop structures were readily interpretable and fell into 3 general categories. These are: (I) structures containing 5 loops, (II) structures containing one distinct loop separated from a triple-loop configuration, and (III) structures containing 3 separate loops (denoted α, β and γ). Examples of these 26S gene R-loop structures are shown in Fig 2. The percentages of observed 26S gene R-loops falling into the described categories are presented in Table 1.

The data summarized in Table 1 show that more than 36% of 26S gene R-loops

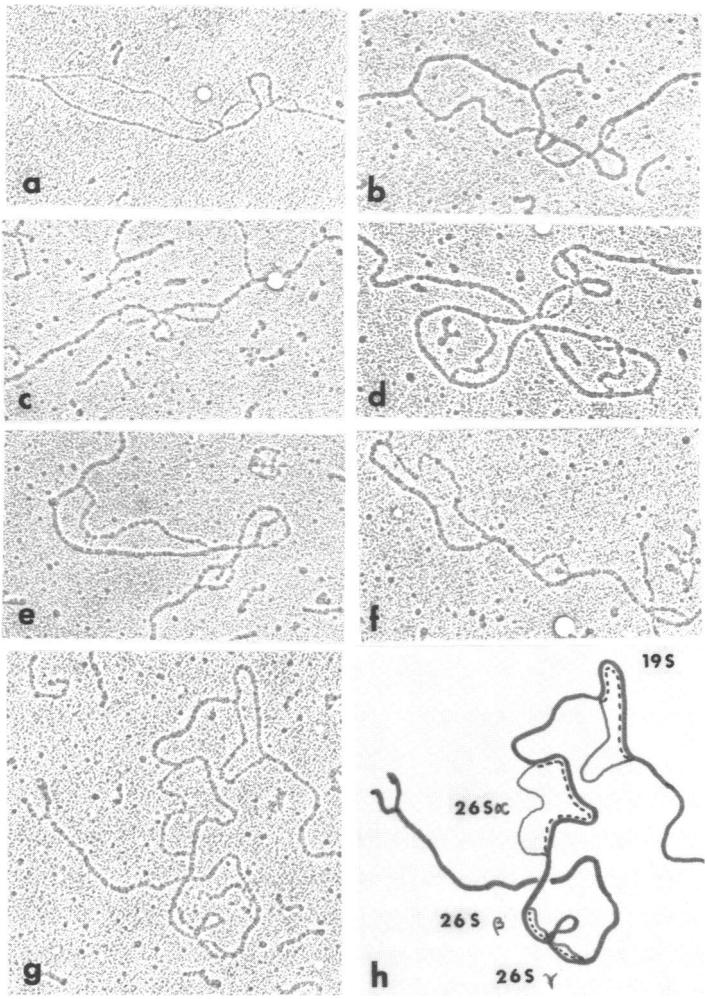


Fig 2. 26S gene R-loop Structures. a,b: 5-loop structures. c,d: structures containing a 3-membered β-γ composite loop. (Note that panel d also includes the 19S gene.) e,f: structures showing the 3 separate 26S gene coding segments and 2 intervening sequences. Segment α is the largest loop. g: one end of the rDNA palindrome showing 19S and 26S coding regions, represented graphically in h. Note the secondary structure at the end of the molecule. Hybridization was conducted using 19S + 26S rRNA as described in Methods. Each panel x 45,000.

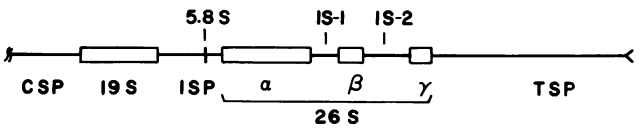
include triple-loop structures such as those in Fig 2 c,d and g, and illustrated in the line drawing in Fig 2. β-γ loops predominate among these category II structures. The type of structure represented in this category is typical

Table 1. Molecular Frequencies in an Annealed Mixture of Purified rDNA and 26S rRNA

Class	Class frequencies	
	No. of ends	% of R-loops scored
I. 5-loop structures	31	23.8
II. Triple-loop structures		
a. α-β	12	9.2
b. β-γ	48	36.9
III. 3 separated loops	24	18.5
IV. <3 separated loops (Incomplete hybridization)	15	11.5
V. No R-loops	13	--
	Σ = 143	

of hybridization of RNA to a DNA coding region which contains an intervening sequence not represented in the hybridizing probe (22). Category III structures, shown in Fig 2, e and f indicate the presence of 26S genes composed of 3 separate coding segments interrupted by 2 intervening sequences, termed IS-1 and IS-2. In at least 23% of R-loops, neither of these intervening sequences is represented in the isolated 26S probe, as indicated by the 5-loop structures shown in Fig 2, a and b, and described in Table 1. Fig 3 includes a graphic representation of 26S gene R-loops. It is not possible to discern single and double stranded loop segments in these 5-loop structures, and 2 possible interpretations are depicted. The data in Table 1 indicate that at least 88% of 26S genes from Physarum microplasmodia contain 2 intervening sequences. IS-1 is not represented in 26S rRNA in at least 33% of R-loops. IS-2 is not represented in 26S rRNA in at least 60% of R-loops. Lengths of the 26S gene coding and intervening sequences are presented in Fig 3. It can be seen that the average length of the more central intervening sequence (IS-1) is 0.68 ± 0.13 kb and that of the more distal (IS-2) sequence is 1.21 ± 0.14 kb. When the data are plotted as lengths of each intervening sequence vs number of molecules observed (not shown) distribution of lengths about the respective averages provides evidence for only one discrete size class for each of these intervening sequences.

Restriction mapping of Physarum rDNA. Purified rDNA was digested with several restriction enzymes used either singly or in combination. Following electrophoresis on 1.0% or 1.4% agarose gels, fragments were sized by comparison of mobilities with those of fragments generated by phage λ DNA digested with



	CSP	19 S	ISP	α	IS-1	β	IS-2	γ	TSP
kb	35.64 ±2.08	2.10 ±0.21	1.66 ±0.12	2.41 ±0.33	0.68 ±0.13	0.70 ±0.11	1.21 ±0.14	0.59 ±0.14	5.37 ±0.88

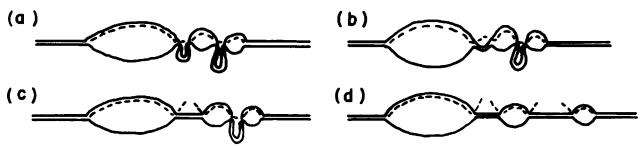


Fig 3. R-loop map of coding sequences at one end of the rDNA palindrome of Physarum. CSP: central spacer. ISP: internal spacer. IS-1 and IS-2: intervening sequences 1 and 2. TSP: terminal spacer. Size averages and standard deviations are based on measurements of >40 molecules in each case. a-d: topography of R-loop structures shown in Fig 2. Physical constraints on forming 5-loop structures (a) may favor DNA reannealing in α or β loops, giving rise to structures such as b.

either Eco R1, Hind III or Hpa I. Standard analyses (c.f. ref. 18) were employed to map positions of restriction cleavage sites.* Sizes and positions of rDNA fragments generated by several restriction enzymes are presented in Table 2. One result of this study is that for each enzyme (excepting Pvu II) the terminal rDNA fragment is visualized as a diffuse band varying in size by ±300 base pairs. In the case of Pvu II, the terminal fragment is too diffuse to visualize readily. This was true whether rDNA was prepared by extraction of nucleolar DNA as described or by selective extraction of rDNA from intact plasmodia (1), which eliminates possible DNase activity. Also note in Table 2 that no band size is given for a Hpa I segment extending from 17.9 to 21.2 kb from the ends of the molecule. This segment is digested to multiple fragments less than 0.2 kb in length, as measured by polyacrylamide gel electrophoresis.

*In the interest of brevity mapping data are not presented here, but are available upon request.

Sizes and Coordinates of Physarum rDNA Restriction Fragments

Enzyme	a	b	c	d	e	f	g	h
Eco R1	7.6-53.4 (45.8)	0 - 5.3 (5.3*)	5.3- 7.6 (2.3)					
Hind III	13.0-48.2 (35.2)	0 - 8.0 (8.0*)	8.0-13.0 (5.0)					
Bam H-I	2.7-23.9 (21.2)	23.9-37.3 (13.4)	0 - 2.7 (2.7*)					
Pst I	15.9-45.2 (29.3)	5.3-15.9 (10.6)	0 - 3.0 (3.0*)	3.0- 5.3 (2.3)				
Hpa I	8.0-17.9 (9.9)	21.2-27.1 (5.9)	27.1-30.3 (3.2)	0 - 3.0 (3.0*)	6.1- 8.0 (1.9)	3.0- 4.7 (1.7)	4.7- 6.1 (1.4)	
Bgl II	12.5-48.7 (36.2)	0 - 9.0 (9.0*)	9.0-12.5 (3.5)					
Xho I	17.0-44.1 (27.1)	0 - 5.9 (5.9*)	8.0-11.1 (3.1)	12.6-14.7 (2.1)	5.9- 8.0 (2.1)	11.1-12.6 (1.5)	15.8-17.0 (1.2)	14.7-15.8 (1.1)
Sal I	17.3-43.8 (26.5)	5.9-12.2 (6.3)	0 - 5.9 (5.9*)	12.2-15.0 (2.8)	15.0-17.3 (2.3)			
Pvu II	12.9-48.2 (35.3)	0.5- 8.6 (8.1)	9.5-12.9 (3.4)	8.6- 9.5 (0.9)	0 - 0.5 (0.5*)			

Table 2. Upper entries are distances from either end of the rDNA molecule to the ends of the fragment. Numbers in parentheses are sizes of fragments in kb (kilobase pairs of DNA).

*Polydisperse band, corresponding to the terminal rDNA fragment. Size given is an average, and the band spans ±0.3 kb from this average.

It is also notable that the restriction cleavage sites of the enzymes tested occur in clusters near the transcription units of the palindrome. Four of the enzymes used do not cleave at all in the central spacer region even though this spacer is 35 kb long.

Hybridization mapping of Physarum rRNA coding regions. The rDNA restriction fragments were transferred to membrane filters (19) and hybridized to ^{32}P-labeled 5.8S, 19S or 26S rRNA species. Representative autoradiographs of several different hybridization experiments are presented in Fig 4. Mapping of the three rRNA coding regions to the rDNA molecule was performed on the basis of these results according to standard procedures. Because the 26S RNA hybridizes to both the Pst I d fragment and the Pvu II c fragment (Fig 4), the length of the 26S gene may be as long as 6.4 kb--considerably longer than the approximately 4.1 kb required to code for 26S rRNA. These data are consistent

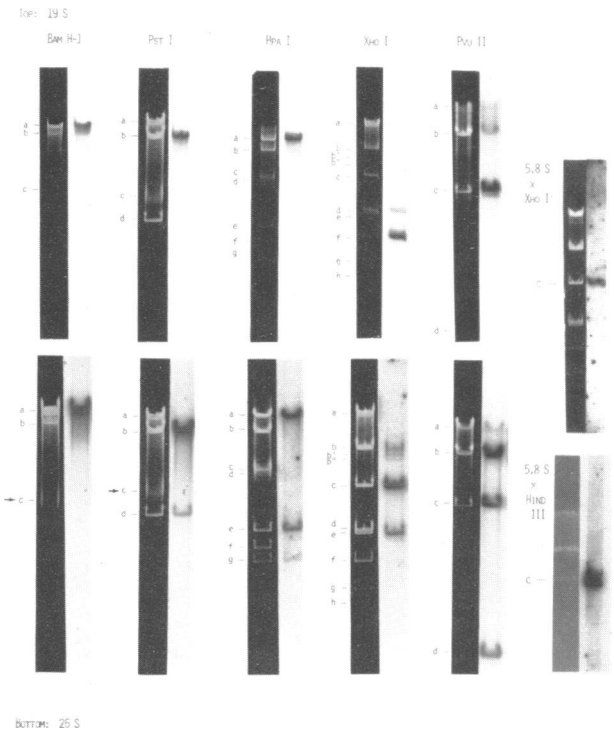


Fig 4. Hybridization mapping of rRNA coding regions. Purified Physarum rDNA was digested with restriction enzymes and resulting fragments hybridized with 5.8S, 19S or 26S rRNAs as described in Methods. Tracks on the left are stained gel bands, and those on the right are autoradiographs of hybridized bands on membrane filters. Arrows in the bottom panel indicate diffuse bands of Bam H-1 and Pst I fragments containing the rDNA termini.

with our R-loop mapping studies showing the presence of 2 26S gene intervening sequences. Restriction fragments produced by Hind III, Pst I, Hpa I or Pvu II containing 26S gene sequences display no length heterogeneity. Thus, there is no evidence for subsets of 26S genes containing discrete but different lengths of intervening sequences, such as have been found in Drosophila (22-24). Heterogeneity is evident in the Xho I b fragment, which hybridizes to 26S RNA (Fig 4). Considered in view of the other data in Fig 4, notably the Hpa I tracks, this is likely to be due to heterogeneity in location of a distal Xho I cleavage site occurring very near the rDNA terminus.

Mapping of the 5.8S gene (Fig 4) indicates that this coding region lies between the 19S and 26S coding regions, as is also true in several other eukaryotes (17, 23,25-27). The labeled 5.8S RNA hybridized exclusively with bands Hind III c and Xho I c in Fig 4. The results do not presently allow us to precisely position the 5.8S gene within the internal spacer. A detailed map of the Physarum ribosomal genes, consistent with both our R-loop and restriction mapping results, is presented in Fig 5. R-loop mapping and restriction mapping results are in good agreement: slight discrepancies between the maps shown in Figs 3 and 5 are within the standard deviations presented in Fig 3.

Secondary structure and length heterogeneity at the rDNA termini. Incubation of rDNA molecules at 45° in 80% formamide induces formation of regions of

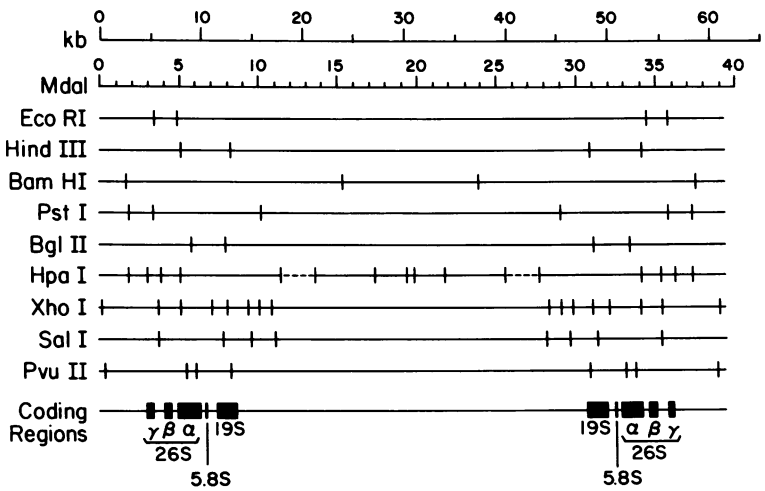


Fig 5. Restriction map of the rDNA palindrome of Physarum polycephalum. One Hpa I region (dotted lines) is cleaved into multiple small fragments which have not been sized. Sizes of restriction fragments are presented in Table 2, and hybridization mapping results are shown in Fig 4.

secondary structure at the rDNA termini, detected as branching in electron micrographs of spread rDNA molecules (Figs 1 and 2). Approximately 30% of identifiable ends possess such structures. Nearly 80% of branched ends are bifurcated, as seen in the bottom panel of Fig 2, and the remainder are trifurcated, as seen in Fig 1. Such regions of secondary structure are not seen when the rDNA is spread directly in 50% formamide without prior heating. Nor has secondary structure of single stranded ends been noticed when rDNA is completely denatured and allowed to fold back (data not shown). The rDNA segment distal to the 26S gene comprises 5.37 ± 0.88 kb as measured electron microscopically. This length variability is higher than that observed for other rDNA regions (Fig 3) and, allowing for a measurement error of <10%, is consistent with a terminal heterogeneity of ±300 base pairs, as observed in restriction enzyme digests (Fig 4).

DISCUSSION

The sequence organization of Physarum ribosomal genes differs substantially from that of several other eukaryotes studied thus far. The Physarum 26S gene includes 2 intervening sequences which are both present in at least 88%--and possibly all--of the 200 gene copies present per plasmodial haploid genome. In contrast, the 25S gene of another lower eukaryote, Saccharomyces cervisiae, contains no intervening sequences at all (17). About 45% of Drosophila 28S rRNA genes contain one intervening sequence, which varies in length, while the remaining 55% are unbroken or continuous genes (23). It has recently been reported that several strains of Tetrahymena pigmentosa possess 25S genes with a single intervening sequence (28). In certain species, including Drosophila (24) and Leishmania (29), 28S genes are interrupted by a DNA "gap" which corresponds to a hidden break in the 28S rRNA. There is at present no evidence for such a break in the Physarum 26S rRNA. The presence of 5-loop structures, as shown in Fig 2, a and b, indicate that neither of the intervening sequences correspond to a break in a substantial percentage of hybridizing 26S rRNA molecules. In addition, we have not been able to detect significant dissociation of the 26S rRNA upon heating to 60° in 80% formamide followed by formamide-sucrose gradient sedimentation or gel electrophoresis. We cannot entirely exclude the possibility that a break in the 26S rRNA exists and is masked by a very tight association of the 2 gene segments.

The plasmodial stage is the most active growth stage of the Physarum life cycle, and the stage at which transcription of most of the ribosomal gene copies

may be expected. In fact, studies on rRNA transcription in isolated plasmodial nuclei suggest that all ribosomal genes are active during this stage (I.Y. Sun, submitted for publication). It is thus likely that both intervening sequences are present in actively-transcribing 26S genes. The RNA complementary to each intervening sequence would then be spliced out of the rRNA primary transcript at some point during processing to the final 26S RNA product. Studies on the primary ribosomal gene transcript, believed to be the 42S rRNA precursor (20, 30), will be necessary to ascertain the extent of transcription of the intervening sequences.

Physarum resembles several other eukaryotes (17, 23, 25-27), including Dictyostelium (26) and yeast (17), with regard to location of the 5.8S gene between the 19S and 26S genes. It is likely that this arrangement is a general feature of eukaryotic rDNA. Physarum differs from certain lower eukaryotes with regard to location of 5S genes. In Dictyostelium (21) and yeast (17) 5S genes are located in the ribosomal gene repeat. However, in Physarum, as in higher eukaryotes, the 5S genes are not linked to the other ribosomal genes (6). It is interesting that in Physarum, Tetrahymena (31, 32) and Dictyostelium (26) the ribosomal genes are linked palindromically while in yeast they are linked in tandem. At present we have only examined one strain on Physarum polycephalum (a x i), and it is possible that intrastrain differences in rDNA structure exist. Our findings indicate that the Physarum rDNA transcription unit must average >9 kb in length. This is in agreement with data of Grainger and Ogle (7), who, using strain M_3CVIII, noted an unusually long ribosomal gene transcription unit (>4 μm) in transcribing chromatin in situ.

Both electron microscopy and restriction mapping indicate that the rDNA segments distal to the 26S gene are heterogeneous in length. Sizing of restriction cleavage products (Table 1) demonstrates that the rDNA terminal fragments vary by ±300 base pairs. The diffuse terminal restriction fragments produced by Bam H-I and Pst I are indicated by arrows in Fig 5. Polydispersity of the terminal Eco R1 fragment has previously been noted (9). The Pvu II cleavage map indicates that this heterogeneity is located within 0.6 kb of the ends of the molecule. This variability is similar in magnitude to the terminal fragment heterogeneity in the Tetrahymena rDNA palindrome, described by Blackburn and Gall (33). We find that regions of secondary structure form at the rDNA termini during heating in formamide, as occurs either during R-loop hybridization or when rDNA is similarly incubated in the absence of RNA. Such branching could be due to denaturation of A-T rich regions at the termini followed by

folding back of inverted repeats. Alternatively, kinking at termini could be a manifestation of single-strand segments at the ends. Such single-strand ends have not previously been detected in rDNA electron micrographs prepared in the absence of formamide (8). Their formation upon heating in formamide could conceivably be facilitated by a loss of small DNA fragments created by nicks in one strand near the terminus of the rDNA duplex. Multiple single-strand nicks have been observed near the Tetrahymena rDNA termini (33). It will be of interest to examine the DNA sequence at the termini of the rDNA palindrome, as well as any possible role of these termini in chromosomal insertion of rDNA sequences during the Physarum life cycle.

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ABBREVIATIONS

rDNA: ribosomal DNA including the ribosomal genes and associated transcribed and nontranscribed spacers. rRNA: ribosomal RNA. kb: as applied to double-stranded DNA, denotes kilobase pairs, 10^3 base pairs. Mdal: 10^6 Daltons.

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