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Sequence arrangement of the 16S and 26S rRNA genes in the pathogenic haemoflagellate Leishmania donovani

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ABSTRACT

Kinetic and chemical analysis show that the haploid genome of Leishmania donovani has between 4.6 and 6.5 x 10^7 Kb pairs of DNA. Cot analysis shows that the genome contains 12% rapidly reassociating DNA, 13% middle repetitive DNA with an average reiteration frequency of 77 and 62% single copy DNA. Saturation hybridization experiments show that 0.82% of the nuclear DNA is occupied by rRNA coding sequences. The average repetition frequency of these sequences is determined to be 166. Sedimentation velocity studies indicate the two major rRNA species have sedimentation values of 26S and 16S, respectively. The arrangement of the rRNA genes and their spacer sequences on long strands of purified rDNA has been determined by the examination of the structure of rRNA:DNA hybrids prepared for electron microscopy by the gene 32-ethidium bromide technique. Long DNA strands are observed to contain several gene sets (16S + 26S). One repeat unit contains the following sequences in the order given: (a) A 16S gene of length 2.12 Kb, (b) An internal transcribed spacer (Sp1) of length 1.23 Kb, which contains a short sequence that may code for a 5.8S rRNA, (c) The 26S gene with a length of 4.31 Kb which contains an internal gap region of length 0.581 Kb, (d) An external spacer of average length 5.85 Kb.

INTRODUCTION

The arrangement of the DNA sequences coding for the stable, large ribosomal RNA species (18S + 28S) has been determined for a limited number of eucaryotic organisms. In the free living protistas examined, Tetrahymena (6), Physarum (7) and Dictyosthelium (8,9), the rRNA genes are arranged in a head to head palindromic array. This organization is different from that observed in the more complex eucaryotic organisms Xenopus and Drosophila where the rRNA genes form a head to tail tandem repeat (10-18).

In view of the observed difference in the organization of rDNA in the three free-living protista and the more complex eucaryotes, we examined the sequence arrangement of rDNA in a parasitic protist, the pathogenic haemoflagellate Leishmania donovani.

We report here our studies of the genome complexity and, the size, number and sequence arrangement of rRNA genes in Leishmania donovani. The genome complexity was studied by standard DNA hybridization techniques (19,20).

The distribution of rRNA coding sequences in enriched nuclear DNA was determined by electron microscope observations of rRNA:rDNA hybrid regions on long single strands of purified rDNA. The hybrid molecules were prepared for microscopy by the gene 32-ethidium bromide (Etd Br)[2] spreading technique (21) which gives excellent discrimination between single-stranded regions and RNA:DNA hybrid regions. The arrangement of rRNA genes was studied by first enriching double-stranded DNA containing rRNA genes from total L. donovani DNA by a modification of the avidin-biotin affinity chromatography method (22,23). We have found that the position and length distribution of rRNA gene sequences and spacer sequences in L. donovani rDNA are similar to the tandem repeat arrangement observed in both Xenopus and Drosophila rDNA.

MATERIALS AND METHODS

Culturing of Organisms

L. donovani Malakal area Sudan strain (19) was the generous gift of Dr. S. Krassner. Culture forms (promastigotes) were maintained at 26°C in 25 ml Erlenmeyer flasks containing 3.0 ml of a modified HO-HEM with the addition of 0.0001 g/l biotin, and 500 units/l penicillin. Cells used for DNA and RNA extraction were grown in a similar way except that 3 liter Fernbach flasks were used containing 1 liter modified HO-HEM. The cell innoculum for all studies was 2.5×10^6 cells/ml. Upon reaching a titer of approximately 4.0×10^7 cells/ml (i.e., early stationary phase) the cells were harvested by centrifugation at 3,000 g for 20 min, washed twice with SSC (0.15 M NaCl, 0.015 M sodium citrate) and either used immediately for nucleic acid extraction or frozen. Promastigote numbers were determined with a Coulter counter, Model ZBI, as previously described (24).

Preparation of DNA

Non-radioactive L. donovani DNA was extracted from whole cells by the procedure previously described for the extraction of DNA from D. melanogaster nuclei (25). ^{32}P labeled L. donovani DNA was obtained by culturing cells to a titer of 4×10^7 cells/ml in medium containing 2.0 mC/l ^{32}P (New England Nuclear, Boston, Mass.). The specific activity of a typical preparation of ^{32}P labeled DNA was 3.4×10^2 cpm/µg as counted in Aquasol (New England Nuclear). Nuclear DNA was purified from preparations of total cellular DNA (nuclear DNA + kinetoplast DNA) by repeated sedimentation through a 20% sucrose solution as described previously (26).

Preparation of rRNA

Ribosomal RNA containing a ^3H-uridine label was obtained by culturing cells to a titer of 4×10^7 cells/ml in medium containing 2.0 mC/l ^3H-uridine

(70 Ci/mmole). Polyribosomes were isolated and rRNA extracted essentially as described by Manning et al. (22). Briefly, the cell pellet was suspended in 0.01 M $MgCl_2$, 0.01 M Tris, 0.05 M KCl, 0.004 M dithiothreitol, 0.25 M sucrose, 1% triton X-100, pH 7.6, and disrupted by 20 strokes in a Dounce homogenizer at 4°C. Monosomes were then isolated from the lysate and 26S and 16S rRNA was extracted from the monosomes as described previously (22). 26S and 16S rRNA was separated by velocity centrifugation through a 5-20% sucrose gradient. The specific activity of the labeled RNA was 3.2×10^4 cpm/μg as determined by TCA precipitates collected on glass fiber filters and counted in a standard toluene based scintillation solution.

Preparation of nonradioactive rRNA-biotin has been described previously as has the labeling of rRNA with ^{125}I (18).

Isolation of rDNA

Procedures for the isolation of double-stranded DNA fragments which contain the rRNA coding sequences from total L. donovani and D. melanogaster nuclear DNA will be described in detail elsewhere (27). The procedure is similar in principal to that described by Manning et al. (23) for the isolation of specific single stranded DNA fragments. Briefly, the molecule biotin is covalently coupled to rRNA. The biotin-rRNA is then hybridized to double-stranded L. donovani DNA in the presence of high formamide and high sodium ion concentration. These conditions allow the RNA to displace the homologous nucleotide sequence in an internal segment of the DNA, resulting in a structure which has been termed an R-loop (38). Those DNA molecules which form R-loops (i.e. rRNA-biotin:DNA hybrids) are selectively removed from the hybridization mixture by binding to avidin-labeled polymethacrylate spheres. The sphere-hybrid complex is separated from the remaining DNA by equilibrium buoyant density centrifugation in CsCl. Removal of the bound DNA from the polymer spheres is accomplished by enzymatic hydrolysis of the RNA with RNase A. The content of rRNA coding sequences in the bound fraction was determined by hybridization with ^{125}I-labeled rRNA.

Denaturation and Renaturation of DNA Fragments

The experimental methods used for denaturation, reassociation, HAP chromatography and cot curves are as previously described (25).

RNA:DNA Renaturation

RNA:DNA hybrids were prepared for electron microscope mapping studies as follows: (a) 10 μl of double strand rDNA (2 μg/ml) was denatured by addition of 2 μl of 1.0 N NaOH. (b) The mixture was neutralized by addition of 2 μl of 2.0 M Tris HCl and 6 μl of H_2O was added. (c) The single stranded rDNA

was then added to 1 µl of rRNA (1000 µg/ml) and 50 µl of 55% formamide, 0.7 M NaCl, 0.1 M Tris, 0.01 M EDTA (pH 8.0) and dialyzed against 55% formamide, 0.7 M NaCl, 0.1 M Tris, 0.01 M EDTA (pH 8.0) at 4°C. The nucleic acid mixture was then allowed to associate as previously described (18).

The amount of rDNA in selected gradient fractions was assayed by ^{125}I rRNA as described previously (23). Radioactively labeled ^{125}I rRNA was prepared by a modified procedure of Orosz and Wetmur (29) as described previously (18).

Hybridization of ^{3}H-labeled rRNA to total cellular L. donovani DNA was performed essentially according to Thomas and Tewari (30). 25 mm nitrocellulose filters containing 30 µg of denatured ^{32}P DNA (34 cpm/µg) were prepared. To these a 2.0 ml solution containing 5.0 to 250 µg/ml of ^{3}H rRNA (3.0×10^{4} cpm/µg) in 2 x SSC was added and the filters were incubated for 24 hours at 65°C. Following the incubation, the filters were washed with 1.0 liter of 2 x SSC and treated with RNase A (25 µg/ml) and RNase T_1 (5 units/ml) for 30 min at 37°C. The filters were then washed with 1.0 liter of 2 x SSC, dried and counted.

Preparation for Electron Microscopy

rRNA:DNA hybrid molecules were prepared as described above and dialyzed for 2 hr at 4° against 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.4. The DNA was treated with gene 32 protein and prepared for electron microscopy exactly as described by Holmes et al. (31). Both double stranded and single stranded ϕX174 DNA were used as internal standards.

Chemical Analysis of DNA Content Per Cell

Total cellular DNA was extracted from 9.0×10^{8} promastigotes using the hot perchloric acid procedure described by DeTorres and Pogo (32). The total DNA content in the final perchloric acid extracts was determined to be 1.05×10^{8} pg by diphenylamine analysis (33). From these measurements the average amount of DNA per cell was calculated to be 0.116 pg.

RESULTS AND DISCUSSION

1. Reassociation Kinetics of Leishmania donovani DNA

Figure 1 shows the reassociation kinetics of 400 nucleotide fragments of L. donovani nuclear DNA using standard hydroxyapatite $(HAP)^{2}$ reassociation conditions. Analysis of the reassociation profile shows the presence of at least three DNA sequence components. These components differ in reiteration frequency and in amounts as follows:

- a highly repetitive component occupying 12% of the genome
- a middle repetitive component $(MR)^{2}$ occupying 13% of the genome

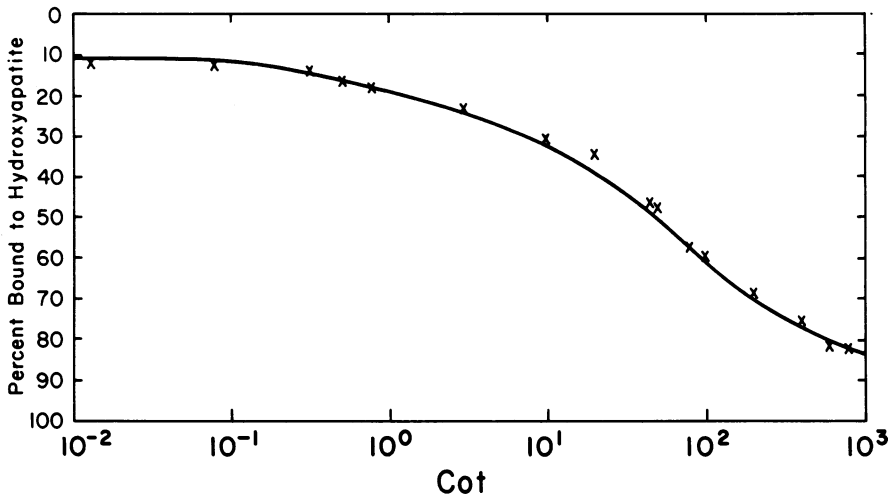


Figure 1. Cot analysis of the reassociation of total cellular L. donovani DNA. The DNA was sheared to an average single-stranded fragment size of 400 bases and renatured under standard hydroxyapatite conditions. The line represents the best fit, by a least squares analysis, of the data.

with a reassociation rate in whole DNA of 1.17 $M^{-1}S^{-1}$ and a repetition frequency of 77

- a putative single copy component, 62% in amount, with a reassociation rate in whole DNA of .016 $M^{-1}S^{-1}$.

The 12% component which we ascribe as highly repetitive sequences has a reassociation rate too fast to be accurately measured in these experiments. However, based on more detailed studies in other organisms, it is likely that this component contains both foldback sequences (34-36) and highly repetitious simple sequences (37). That fraction of the DNA which does not reassociate (10-15%) has been determined, by sedimentation velocity studies, to be considerably degraded.

The major fraction of the genome (62%) is occupied by a component which reassociates at a rate suggestive of single copy sequences. It has been shown that the haploid genome size of an organism can be determined by a comparison of the reassociation rate of single copy sequences in the total DNA and the rate constant of a kinetic standard (19,20). Under the reassociation conditions used in these experiments, the rate constant for 400 nucleotide fragments of E. coli DNA is 0.25 $M^{-1}S^{-1}$. The genome of E. coli contains 4.2×10^6 nucleotide pairs of DNA (40). The rate constant for the presumed

single copy sequences in L. donovani nuclear DNA is 0.016 $M^{-1}S^{-1}$. Based upon this measurement, the haploid genome contains 6.5 x 10^7 nucleotide pairs of DNA.

Chemical analysis of total cellular L. donovani DNA yields 0.116 pg DNA/cell. Since approximately 15% of the total cellular DNA in L. donovani is found in the kinetoplast [3], 0.10 pg of the total cellular DNA can be considered nuclear DNA and .016 pg kinetoplast DNA. Using 6.6 x 10^2 daltons/nucleotide pair, the number of nucleotide pairs of DNA per nucleus based on chemical analysis can be calculated to be 9.13 x 10^7 $\left(\frac{1.0 \times 10^{-13} \text{ g/nucleus}}{6.6 \times 10^2 \text{ g/mole nucleotide pairs}} \times \frac{6.023 \times 10^{23} \text{ nucleotide pairs}}{\text{mole nucleotide pairs}}\right)$. This value is in reasonable agreement with the 1.3 x 10^8 nucleotide pairs/nucleus (6.5 x 10^7 nucleotide pairs/haploid genome x 2) expected for a diploid genome based on our kinetic studies. We conclude, therefore, that the most slowly reassociating sequences in L. donovani nuclear DNA do represent single copy sequences, and that the haploid genome size based on chemical and kinetic analysis is between 4.6 and 6.5x10^7 nucleotide pairs.

2. Analysis of L. donovani rRNA by velocity sedimentation

Optical quantities of L. donovani total cellular RNA was mixed with ^{3}H-uridine labeled D. melanogaster rRNA from tissue culture. The mixture was then cosedimented in a 5 to 20% linear sucrose density gradient. The results, shown in Fig. 2, indicate that the major L. donovani rRNA species sediment at approximately 26S and 16S, respectively. These results are in substantial agreement with earlier studies[4] on the size of the RNA in another species of kinetoplastidia, Crithidia acanthocephali. It should be noted, however, that the sedimentation value obtained in these studies as well as those of Fouts and Wolstenholme for the small ribosomal RNA (16S) is substantially less than the 21S value reported earlier for the small rRNA in the kinetoplastidia, Tryponosoma cruzi (39).

3. Hybridization of rRNA

The portion of the nuclear DNA homologous to rRNA was determined by rRNA/DNA saturation hybridization experiments. As shown in Figure 3, rRNA hybridizes at saturation to 0.83% of the nuclear DNA. Using a nuclear haploid genome size of 6.5 x 10^7 nucleotide pairs, and the sum of the molecular lengths of the 26S and 16S Leishmania rRNA to be 6.41 Kb (see below), the number of rRNA gene sets (26S + 16S) in the L. donovani haploid genome is about 166. Solution hybridization experiments using very large excesses of rRNA to rDNA (900:1) gave results which agree with those of the DNA filter experiments as to the number of ribosomal RNA gene sets present in the genome (data not shown).

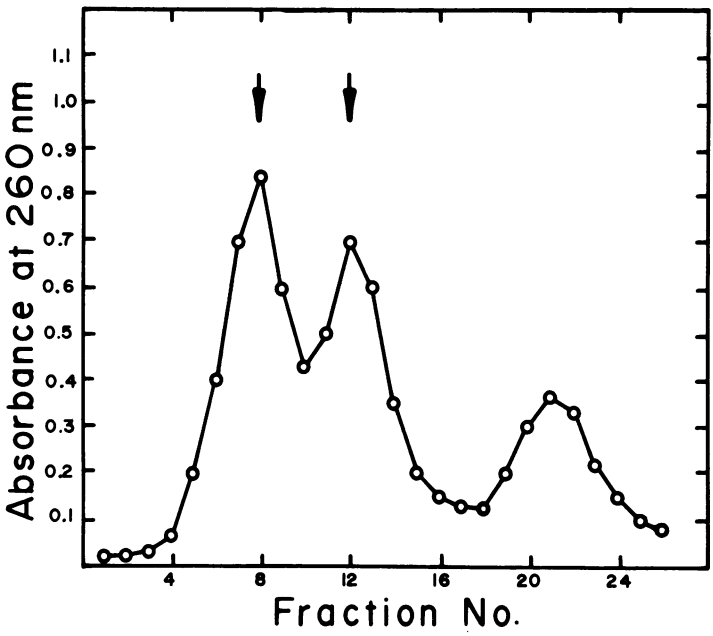


Figure 2. The distribution of total cellular L. donovani RNA in a linear 5% to 20% sucrose sedimentation gradient. The arrows represent the positions of the two major components of cosedimented ^{3}H-uridine labeled 26S and 16S rRNA from D. melanogaster. Sedimentation was from right to left.

4. The Arrangement of rDNA

The enrichment of double stranded rDNA from total L. donovani DNA is briefly described in Materials and Methods and will be described in detail elsewhere (27). The strands of rDNA used in the studies reported here were 28% pure and had a double stranded weight average length of 90 Kb and a single stranded weight average length of 29 Kb. The arrangement of rRNA coding sequences on these strands is shown in Fig. 4 as a final model for the structure of L. donovani rRNA. A single repeat unit contains the following:

(a) a 16S gene of length 2.12 Kb

(b) an internal spacer (Sp1' + 5.8S + Sp1") of length 1.23 Kb.

(c) an 26S gene of length of 4.31 Kb which contains an internal gap region of .587 Kb.

(d) an external spacer, Sp2, of average length 5.85 Kb.

It may be noted that the two regions denoted 26Sa and 26Sb are similar in size to the region we describe as the 16S gene. It is possible, therefore, based solely on length measurements that the gene order may be different than

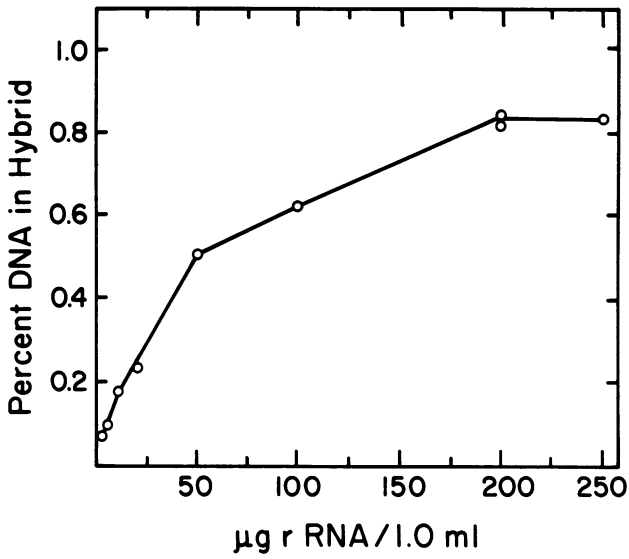


Figure 3. The results of filter hybridization experiments of denatured L. donovani total cellular DNA with increasing amounts of ^{3}H-uridine labeled L. donovani rRNA. Incubation was with 30 µg of DNA on nitrocellulose filters in 2 X SSC at 65°C for 24 hrs with the amounts of rRNA indicated. The filters were treated with RNase, washed extensively, dried and counted. Maximum hybridization was 0.82% of the nuclear DNA.

that shown in figure 4 and may be represented by one of the following:

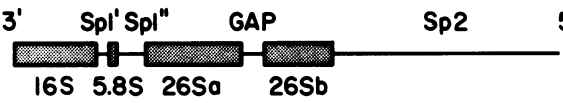
(a) 26Sa(or b) - 5.8S - 26Sa(or b) - 16S

(b) 26Sa(or b) - 5.8S - 16S - 26Sa(or b)

To determine if either of these gene arrangements were present in the rDNA, 26S rRNA (twice purified by sucrose gradient centrifugation) was hybridized to enriched rDNA. Of the molecules examined (N=40) only hybrid structures consistent with the model depicted in figure 4 were observed. No hybrid structures consistent with either model a or b were observed. We conclude, therefore, that the arrangement of the 26S gene is as shown in figure 4. If this is the case, then the respective rRNA gene sequences may only be arranged as shown in figure 4.

The presumed 3' to 5' polarity of the coding strand of rDNA indicated in the figure is assigned from the recent determination of the polarity of the 40S rRNA precursor of Xenopus (40).

Figure 5a is an electron micrograph showing the repeat unit on a single strand of rDNA after hybridization with 16S and 26S rRNA and preparation for electron microscopic observation by the gene 32-Etd Br procedure. This mole-



16S	Spl'	5.8S	Spl"	26Sa	GAP	26Sb	Sp2
2.12	0.33	0.20	0.70	1.94	0.59	1.78	5.85
±0.23	±0.08	±0.04	±0.10	±0.21	±0.13	±0.20	±0.82
(54)	(42)	(42)	(42)	(41)	(41)	(42)	(47)

Figure 4. A schematic diagram of a repeat unit of rDNA from L. donovani. The 5.8S, 16S and 26S gene regions are depicted by heavy bars while the spacer sequences (Sp) and the "Gap" sequences are denoted by thin lines. The lengths (in kilobases) of all sequences are shown below the diagram. The number of molecules measured of each sequence is shown in parentheses. The 3' to 5' designation in the diagram refers to the presumed polarity on the coding strand of rDNA (see text).

cule shows the structural features of the rDNA repeat unit which are discussed in detail below.

The Internal Spacer and 5.8S Gene

The short spacer region separating the 16S and 26S genes consistently contains a short duplex region as shown in the electron micrograph of Fig. 5b. It has previously been found in Xenopus rDNAs (42) that this short spacer region is transcribed and contains the 5.8S rRNA gene. More recent studies on Drosophila rDNA indicate that the gene coding for 5.8S rRNA is also found within this short spacer region (18). These results have recently been confirmed by studies by Glover and Jordon[5] on the 5.8S and 2.0S Drosophila rRNA's. Since both the size and position of the small duplex region observed in Leishmania rDNA is like that found in Xenopus and Drosophila rDNA, we presume that this RNA:DNA hybrid region is due to the presence of a gene which codes for an RNA similar in nature to the 5.8S RNA found in those organisms.

The 26Sa and 26Sb Segments

We consistently observe (95% of all rDNA molecules) a short single stranded region of average length 0.58 Kb within the 26S gene region (Fig. 5a) at a point 1.94 Kb from the junction of the 26S gene with Spl". The single stranded region is designated a "Gap" region and the two portions of the 26S gene on either side of the gap are denoted 26Sa and 26Sb (notation of Wellauer and Dawid, 17). The length of the gap, 26Sa and 26Sb regions are

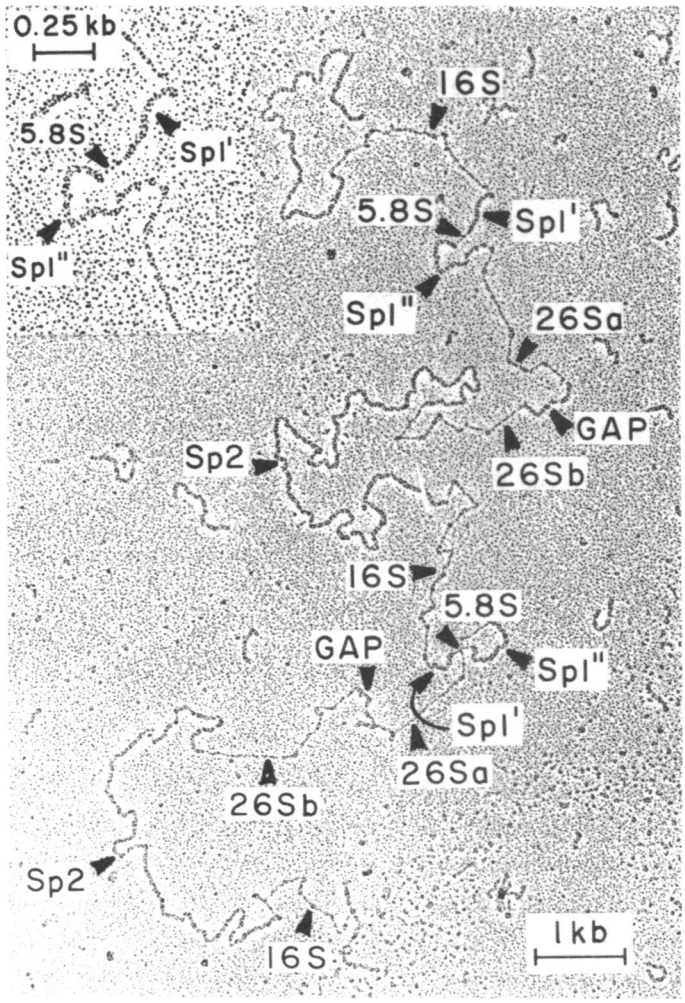


Figure 5. Electron micrographs of rDNA:rRNA hybrid molecules prepared for electron microscopy by the gene 32-protein technique. A. A molecule showing the various features described in the text. B. A high magnification micrograph of the transcribed spacer (Spl) and the 5.8S gene region.

given in Fig. 4. The standard deviations of the length distribution of the 26Sa and 26Sb regions are approximately 10% which is about that expected if each segment is homogeneous in length. We, therefore, conclude that the position of the gap is at a reproducible point within the total 26S gene.

The appearance of a gap region 0.14 Kb in length has been previously observed in the 28S gene region of Drosophila rDNA (17,18). The studies of Wellauer and Dawid (17) position the gap region at a site at which the 26S

Drosophila rRNA is cleaved into two fragments, 28Sa and 28Sb, by a post-transcriptional ribonuclease processing step within the ribosome (43). Interestingly, Wellauer and Dawid observe the gap region to be highly reproducible in the 28S gene region, while Pellegrini et al. (18) observed the gap in only 10% of the 28S gene regions. This suggests that although all 28S rRNA molecules contain a hidden break, only a portion of the molecules have had an observable number of ribonucleotides removed from the initial cleavage site. Recent observations by Jordan[5] suggest that 28S rRNA extracted in the absence of RNase inhibitors or from cells in stationary phase are slightly degraded and have lost several nucleotides around the central hidden break. These observations invite the possibility that L. donovani 26S rRNA, like that in Drosophila, is cleaved by a post-transcriptional ribonuclease processing step and that the large gap observed in the coding sequence represents an RNase sensitive region around the site of the central break. After heating at 60°C for 15 min., followed by sedimentation in a linear 5% to 20% sucrose gradient, L. donovani rRNA was distributed in a single broad peak with a mean sedimen-

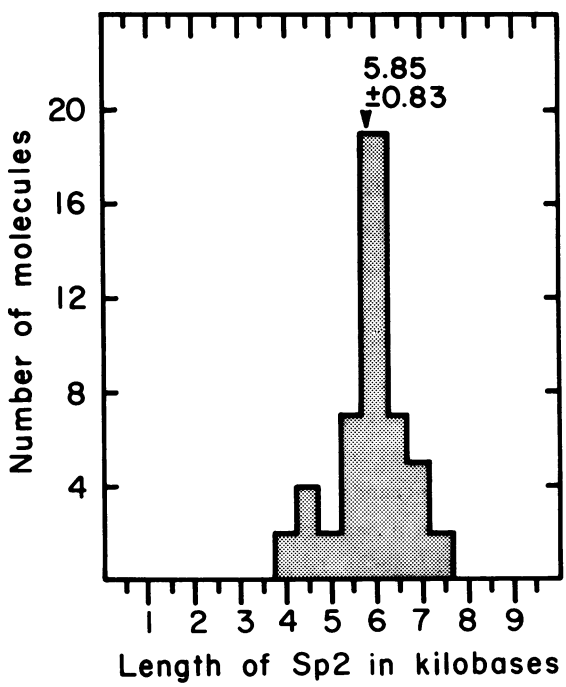


Figure 6. A histogram of the observed distribution of the lengths of the external spacer (Sp2) from L. donovani. The average length of the spacer is indicated by the arrow above the histogram and is given in kilobases with its standard deviation.

tation coefficient of 17S. This result is also consistent with there being a central break in the 26S rRNA molecule.

The External Spacer

The observed length distribution for the external spacer (Sp2) is shown in Fig. 6. The average length is 5.85 Kb with a standard deviation of 0.82 Kb. Although the spacer is somewhat heterogeneous in length, it is clear that it exhibits a far more homogeneous length distribution than that observed for the external spacer in either Drosophila or Xenopus rDNA (17,18).

Further Discussion

Quantitative reassociation experiments and chemical analysis studies indicate that the complexity of L. donovani nuclear DNA is between 4.5 and 6.5 x 10^7 Kb. This value is about the same as that observed for Dictyosthelium (44) and is about 1/3 to 1/2 that for Drosophila DNA (25). It is certainly much lower than that found for many other eucaryotic genomes. The fraction of the genome occupied by middle repetitive sequences (13%) and the reiteration frequency (ca. 77) of these sequences is also low in comparison to other eucaryotes. The possible interspersion of the middle repetitive sequences among single copy sequences in the genome, as has been observed for the arrangement of these sequences in most eucaryotic DNA's (37), remains to be investigated.

The amount of L. donovani nuclear DNA that codes for rRNA is 0.82%. There are, therefore, about 166 copies of the 16S and 26S rRNA genes per haploid equivalent of DNA (see Results). The amount of the genome occupied by the rDNA, and thus the reiteration frequency of the rRNA genes in L. donovani is similar to that observed in the protist Dictyosthelium (44) and Physarum (7). However, the arrangement of the gene sets (16S + 26S) is clearly quite different. In Dictyosthelium (8,9), Physarum (7) and in the macronucleus of Tetrahymena (6) the rRNA genes appear to be arranged on extra-chromosomal DNA in a symmetrical head-to-head or palindrome structure.

In L. donovani long single strands of rDNA contain several sets of rRNA genes (16S + 26S) all arranged in a head-to-tail tandem array. This indicates that the organization of the rRNA genes in L. donovani is unlike that observed in other protists but similar to that observed for the rDNA in the more complex eucaryotes Xenopus (10-13) and Drosophila (14-17). It further suggests that these genes are clustered in long tandem repeats within the genome. Consistent with this interpretation is the observation that rDNA strands which are 3 to 6 times longer than a single repeat unit always contain multiple gene sets. However, whether the clustered gene sets are all localized within one

region of the genome or are found at a few distinct regions on the chromosomes, as has been observed in several eucaryotic organisms (45-48) by in situ hybridization experiments, cannot be determined by this study. It should be noted that the palindromic arrangement for rRNA genes in the above protists may not necessarily be directly correlated with the occurrence of these genes on extrachromosomal DNA. In the case of Xenopus oocytes, the amplified rDNA is extrachromosomal, but is arranged in a head-to-tail tandem array (49).

Although the organization of rRNA genes has been determined in only a small number of eucaryotic DNAs, it appears that certain common features exist for the structural arrangement of these genes. In both simpler and more complex eucaryotic organisms the 18S and 28S coding sequences are repeated several hundred fold per cell and are contained within repeat units that include discrete spacer sequences. These similarities invite the interesting speculation that an obsolute number of rRNA coding sequences are necessary for normal functioning of a eucaryotic cell, and that the tandem arrangement of these genes may be of functional and/or evolutionary significance.

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