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The organization of the ribosomal RNA genes in the fungus Mucor racemosus

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ABSTRACT

The rDNA of Mucor racemosus is contained on a 6.4 megadalton repeat unit. Two Bam H-1 restriction fragments that encompass the entire rDNA repeat, as well as two Hind III restriction fragments that lie within the region, have been cloned and analyzed. The rDNA unit has been defined with respect to eight restriction endonucleases and the position of the sequences encoding the 25S, 18S, and 5.8S rRNA species have been localized. In addition, the 5S RNA encoding sequence was found to reside within the basic repeat unit. The results indicate the organization of the rDNA of Mucor more closely resembles the arrangement observed in yeast than that observed in other eukaryotic organisms.

INTRODUCTION

Mucor racemosus, a dimorphic phycomycete, can readily be manipulated to grow in either yeast or hyphal morphology by alterating environmental conditions (1-4). The ease with which the transition between these two distinct cell types can be mediated makes Mucor an attractive system in which to study differential gene expression during a simple developmental process.

As a first step in our studies designed to investigate the salient features of the genomic organization of Mucor, as well as those genes subject to differential expression during morphogenesis, we have assembled a Mucor clone bank in Escherichia coli that was constructed by the insertion of Hind III or Bam H-1 restriction fragments of Mucor DNA into the plasmid pBR322. We have begun a systematic screening of these clones to identify those of interest and here report the organization of the genes that specify the ribosomal ribonucleic acids of Mucor (rDNA). It has been determined that the general characteristics of the region more closely resemble the rDNA arrangement of Saccharomyces cerevisiae (5) than the arrangement observed in several other lower eukaryotes.

MATERIALS AND METHODS

Media and Bacterial Strains. YP contained 1% peptone and 0.3% yeast extract at pH 4.5. YPG is YP supplemented with 2% glucose. L broth (6) was supplemented with 40 µg/ml ampicillin for growth of strains harboring recombinant plasmids. L agar contained 1.5% agar.

Ampicillin at 40 µg/ml and tetracycline at 40 µg/ml were present in L agar plates when required for selection. E. coli strain C600 (pBR322) was obtained from Dr. G. Wesley Hatfield. E. coli strain JA221 was obtained from Dr. John Carbon.

DNA Purification. A 14 hour culture of Mucor, maintained under either air or low-flow N_2 (4), was collected by filtration. The mycelial mat obtained was frozen with liquid N_2 and then disrupted by grinding. The still frozen cell debris was placed into 1/10 volume of 10 mM Tris, pH 8.3 containing 150 mM EDTA and 100 mM NaCl and an equal volume of buffer-saturated phenol. After extraction at room temperature for 4-6 hr, phases were separated by centrifugation at 10,000 x g for 10 min and the aqueous phase was removed, transferred into an equal volume of chloroform:isoamyl alcohol ($CHCl_3$:IAA) (24:1) and extracted for 1 hr at room temperature. This was repeated until the interface between the two phases was clear. DNA was then precipitated by addition of 2 volumes of ethanol, spooled, and allowed to solubilize in 0.1X SSC at 4°C overnight. The preparation was treated with RNase at 20 µg/ml for 2 hr, followed by incubation with Proteinase K (2 mg/ml) for 2 hr. Protein was removed by extraction with $CHCl_3$:IAA and DNA was again precipitated with ethanol and resuspended in 1 mM Tris, pH 7.5 containing 0.5 mM EDTA.

Alternatively, after the initial $CHCl_3$:IAA extraction, the crude DNA preparation was subjected to CsCl-ethidium bromide density gradient centrifugation at 80,000 x g for 60 hr. The DNA containing band was removed and the centrifugation was repeated. The DNA containing band was then removed, extracted with CsCl-saturated isopropanol and dialyzed extensively against 1 mM Tris, pH 7.5.

Plasmid DNA was purified by CsCl-ethidium bromide density gradient ultracentrifugation of cleared lysates (7).

Cloning. Mucor DNA and pBR322 DNA at a ratio of 8:1 were digested to completion with either Hind III or Bam H-1. The reaction was stopped by incubation at 65°C for 10 min. The fragments were then ligated as described elsewhere (8). Transformation of JA221 (9) was accomplished by heat shocking $CaCl_2$-treated cells for 3 min at 37°C. Recombinant DNA-containing clones were selected by the amp^r tet^s phenotype of such clones.

RNA Purification. Spores of Mucor (5×10^5 spores/ml) were inoculated

into YPG containing $^{32}PO_4$= at 50 μCi/ml. After 12-14 hr, mycelia were harvested by filtration and disrupted by grinding under liquid N_2. Cell debris was placed into 5 ml cold TNE (10) containing 0.3% diethylpyrocarbonate (DEPC). An equal volume of cold water-saturated phenol was added and the mixture was shaken intermittently during a 15 min incubation on ice. The phases were then separated by brief centrifugation at 10,000 x g and the aqueous layer removed and reextracted with water-saturated phenol. The procedure was continued until no precipitate appeared at the interface. The crude RNA preparation was adjusted to 0.3 M with ammonium formate and 2 volumes of cold ethanol were added. After overnight incubation at -20°C, RNA was precipitated by centrifugation at 14,000 rpm for 20 min, dried under vacuum and resuspended in 0.5 ml ANE buffer (10). The RNA was layered on to a 5-20% sucrose gradient in ANE and centrifuged at 25,000 rpm for 18 hr in tubes appropriate to the SW27.1 rotor. Fractions were collected and those containing 25S, 18S, and soluble RNAs were pooled individually and precipitated as before. The 18S and 25S species were again centrifuged as described above. Peak fractions containing either 18S or 25S RNA were pooled, precipitated and finally resuspended in 0.5 ml sterile distilled water. Purity was assessed by electrophoresis in gels containing 2.5% acrylamide and 0.5% agarose.

Soluble RNA was further purified by electrophoresis as described (11). The position of 5.8S and 5S RNA in gels was determined by autoradiography. The appropriate gel portions were cut out, homogenized, and incubated overnight at 30°C in 2 volumes of 0.1 M Tris-HCl, pH 9.1 containing 0.5 M NaCl and 10 mM EDTA. After two extractions with cold, water-saturated phenol, the eluted RNA was adjusted to 0.3 M ammonium formate, precipitated by addition of 2 volumes of cold ethanol and maintenance at -20°C overnight. The individual RNA species were finally collected by centrifugation and resuspended in sterile distilled water.

Alternatively, ^{32}P-labeled 5.8S and 5S RNA species were extracted from whole cells by the procedure described by Rubin (11), except the temperature for the initial phenol extraction was 45°C. The individual soluble RNA species were then purified as described above.

Hybridization. The modified procedure for colony hybridization (12) was utilized to identify putative rDNA containing clones. Hybridizations were performed in 5 x SSC containing 0.1% SDS for 16 hr at 65°C in heat-sealable bags. Transfer of restriction fragments to nitrocellulose was done as described by Southern (13). Hybridizations were carried out at 42°C in 50%

formamide and 5 x SSC or at 65°C in 5 x SSC containing 0.1% SDS. After hybridization, filters were washed in the hybridization buffer utilized at the appropriate temperature. This was followed by 15 minute washes at room temperature in 2 x SSC, 2 x SSC containing 2 µg/ml pancreatic ribonuclease, and 2 additional washes in 2 x SCC. Filters were then blotted dry, wrapped in plastic wrap, and subjected to autoradiography.

Enzyme Digestion and Electrophoresis. All restriction enzyme digests were performed under optimal conditions described by the supplier. Agarose gel electrophoresis was carried out in gels containing 1.2% agarose and 0.5 µg/ml ethidium bromide for 16 hr at 30V or 4-5 hr at 100V.

Materials. All enzymes were obtained from New England Bio Labs or Miles Laboratories. Agarose was purchased from Bethesda Research Laboratories. Components for polyacrylamide slab gels were from Bio-Rad.

RESULTS

Identification of DNA Restriction Fragments Containing rRNA Genes. The size and number of Hind III and Bam H-1 restriction fragments containing sequences complementary to rRNA were determined by hybridizing ^{32}P-labeled 18S and 25S rRNA to Mucor DNA that had been totally digested with the respective restriction endonuclease, and then transferred from agarose gels to nitrocellulose (13). Results shown in Figure 1 indicate that the 18S and 25S sequences were contained on 3 Hind III restriction fragments of approximately 1.2 Kb, 3.9 Kb, and 5.1 Kb or a combined molecular weight of approximately 6.4 megadaltons. Similarly, 18S and 25S sequences were found on two Bam H-1 restriction fragments of 4.7 Kb and 5.5 Kb with a total molecular weight also of about 6.4 megadaltons.

Colony Hybridization. In order to isolate and further analyze the arrangement of the rDNA containing fragments depicted above, the clone bank was screened for those containing sequences complementary to 18S and 25S rRNAs. Approximately 2-4% of all clones constructed were preliminarily identified as containing at least a portion of the 18S and 25S region. After isolation and purification of the recombinant plasmids from several of the putative rDNA-containing clones, they were further characterized as to the specific Bam H-1 or Hind III rDNA insert present by digestion with the appropriate restriction endonuclease and analysis on agarose gels. Transfer of the fragments to nitrocellulose and hybridization to ^{32}P-labeled 18S and 25S rRNA definitively identified the 1.2 Kb and 3.9 Kb Hind III rDNA fragments as well as both Bam H-1 fragments (data not shown). Thus,

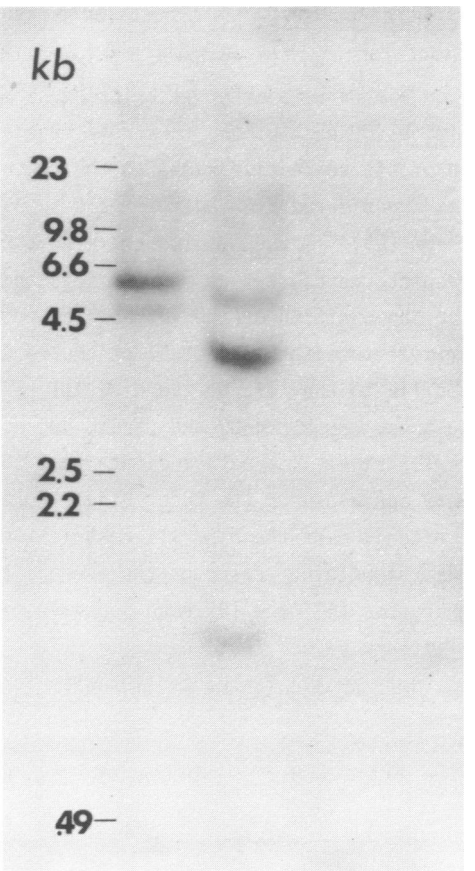

FIGURE 1
The number of Mucor DNA fragments containing rDNA sequences. Mucor DNA totally digested with either Bam H-1 or Hind III was subjected to electrophoresis in a 1.2% agarose gel and transferred to nitrocellulose by the method of Southern (13). The autoradiograph depicts hybridization of ^{32}P-labeled 18S and 25S rRNA to: Lane 1 Bam H-1 restriction fragments; Lane 2 Hind III restriction fragments.

clones containing recombinant plasmids encompassing the entire rDNA region were isolated.

Localization of the 18S and 25S Genes. The two Bam H-1 generated recombinant plasmids designated, pMu294 and pMu300, and the two recombinant plasmids containing Hind III-generated inserts, designated pMu37 and pMu438, were subjected to digestion with a number of restriction endonucleases in

order to establish the restriction map of the rDNA region presented in Figure 2. A Bam H-1 restriction site, assigned to the left end of the pMu300 Mucor insert, serves as the "0" reference point in the map from which the distance in megadaltons to other restriction sites was determined.

The relative positions of each of the four rRNA species have been determined by hybridization of the respective rRNAs to the restriction fragments of the cloned sequences transferred from agarose gels to nitrocellulose by the method of Southern (13). These results are also summarized in Figure 2.

Figures 3 and 4 depict representative agarose gels and hybridization patterns used to define the position of the 18S and 25S encoding sequences. 18S rRNA does not hybridize to either the pMu37 or pMu294 fragment and thus, the 18S gene must lie to the left of the Hind III restriction site located at 1.95 megadaltons. Both pMu300 and pMu438 do, however, contain sequences that specify 18S rRNA. The data indicate that the 18S encoding sequence is not entirely contained on the Mucor Hind III restriction fragment of pMu438, but rather extends past the Hind III restriction site located at 1.2 megadaltons towards the origin. Based on the average size of the 18S RNA of other lower eukaryotes (14) the 18S rDNA sequence most likely continues an additional 1.0 Kb leftward beyond that point.

On the other hand, only pMu438 failed to hybridize to 25S RNA, indi-

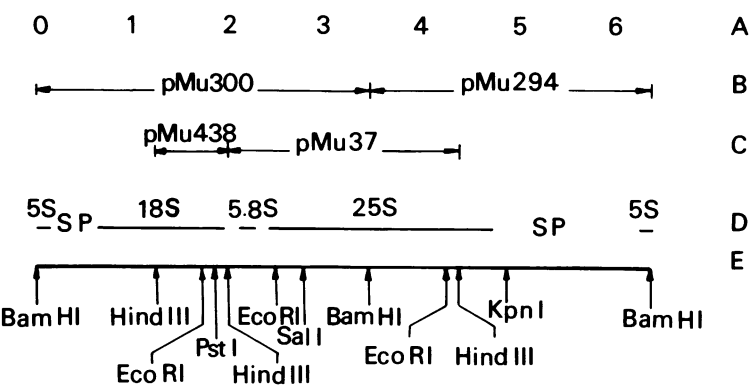

FIGURE 2
Organization of the rRNA gene cluster of Mucor racemosus. Line A represents the distance across the region in megadaltons. Lanes B and C define the limits of the four cloned rDNA segments. Line D shows the relative position of the coding sequences of the four rRNA species. Line E shows the restriction map of the region. Besides the enzymes listed, it was determined that there were no cleavage sites in the unit for Bgl I and Pvu II. For details refer to the text. (Spacer sequences = SP.)

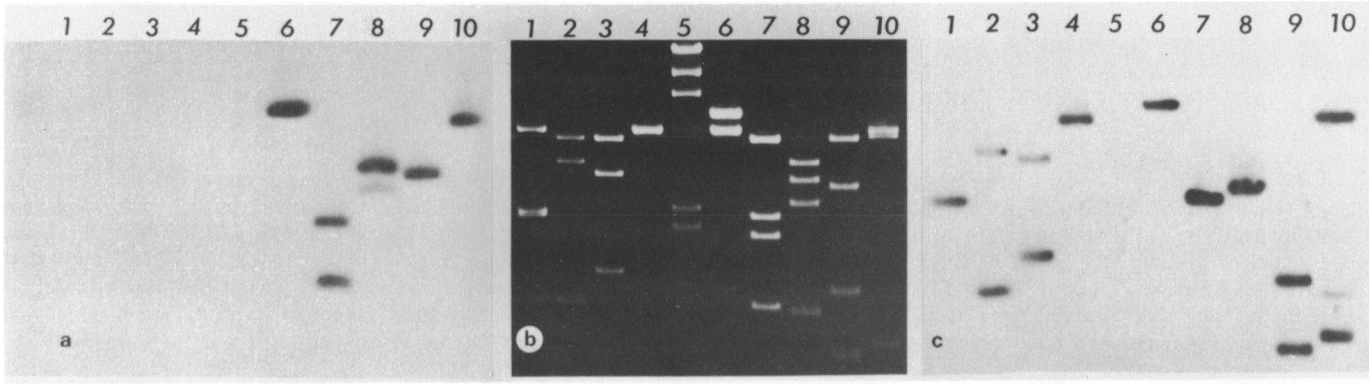

FIGURE 3

Position of the 18S and 25S encoding sequences. (b) Plasmids pMu294 and pMu300 were digested with the appropriate restriction endonucleases and subjected to electrophoresis through a 1.2% agarose gel and stained with ethidium bromide. Lanes 1-4 pMu294 digested with: Lane 1, Bam H-1 and Kpn I; Lane 2 Bam H-1 and EcoR1; Lane 3, Bam H-1 and Hind III; Lane 4, Bam H-1; Lane 5, λDNA digested with Hind III; Lane 6-10, pMu300 digested with: Lane 6, Bam H-1; Lane 7, Bam H-1 and Hind III; Lane 8, Bam H-1 and Pst I; Lane 9, Bam H-1 and EcoRI; Lane 10, Bam H-1 and Sal I. (a) Autoradiograph of the pMu294 and pMu300 restriction fragments after hybridization to ^{32}P-labeled 18S rRNA. (c) Autoradiograph of the pMu294 and pMu300 restriction fragments after hybridization to ^{32}P-labeled 25S rRNA.

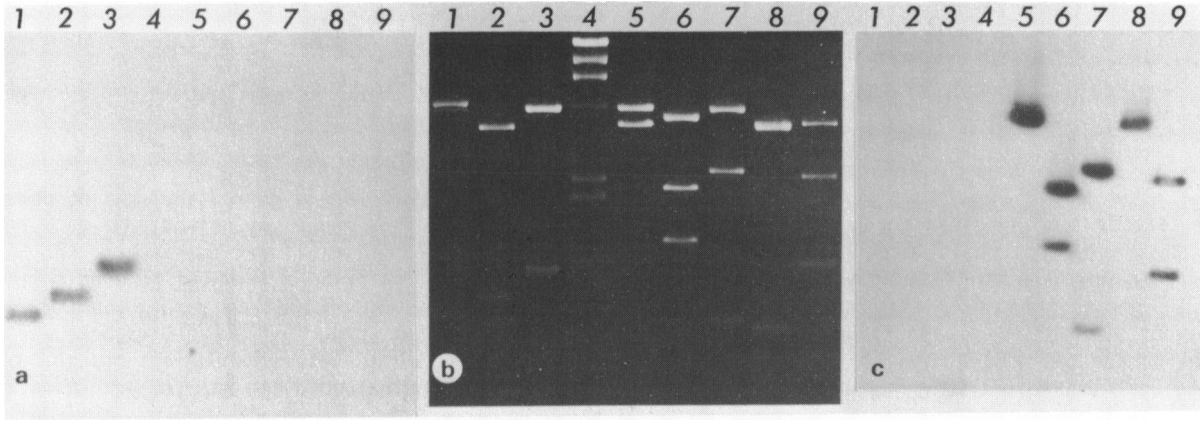

FIGURE 4

Position of the 18S and 25S encoding sequences. (b) Plasmids pMu37 and pMu438 were digested with the appropriate restriction endonucleases and subjected to electrophoresis through a 1.2% agarose gel and stained with ethidium bromide. Lanes 1-3, pMu438 digested with: Lane 1, Hind III and EcoRI; Lane 2. Hind III and Pst I; Lane 3, Hind III; Lane 4, λ digested with Hind III; Lanes 5-9, pMu37 digested with: Lane 5, Hind III; Lane 6, Hind III and Bam H-1; Lane 7, Hind III and EcoRI; Lane 8, Hind III and Pst I; Lane 9, Hind III and Sal I. (a) Autoradiograph of the pMu37 and pMu438 restriction fragments after hybridization to ^{32}P-labeled 18S rRNA. (c) Autoradiograph of the pMu37 and pMu438 restriction fragments after hybridization to ^{32}P-labeled 25S rRNA.

cating that the 25S encoding sequence must begin to the right of the Hind III restriction site positioned at 1.95 megadaltons. Again, based on the reported size averages of 25S RNA in lower eukaryotes (13), the most likely termination point lies between the Hind III restriction site and the Kpn I restriction site at 4.85 megadaltons.

Position of the 5.8S and 5S RNA Encoding Sequences. Hybridization of the 5.8S rRNA species to restriction fragments generated from plasmids pMu37 and pMu438 (Figure 5) indicated that the 5.8S sequence was contained in the region bounded by the Hind III restriction site at 1.95 megadaltons and Eco Rl restriction site at 2.5 megadaltons. This result also implies that the start of the 25S sequence is at least 0.25 megadaltons rightward from the Hind III restriction site at 1.95 megadaltons in order to allow for positioning of the 5.8S RNA species. Hybridization of the 5S RNA species to plasmids pMu294 and pMu300 (Figure 6) revealed that the 5S rDNA region

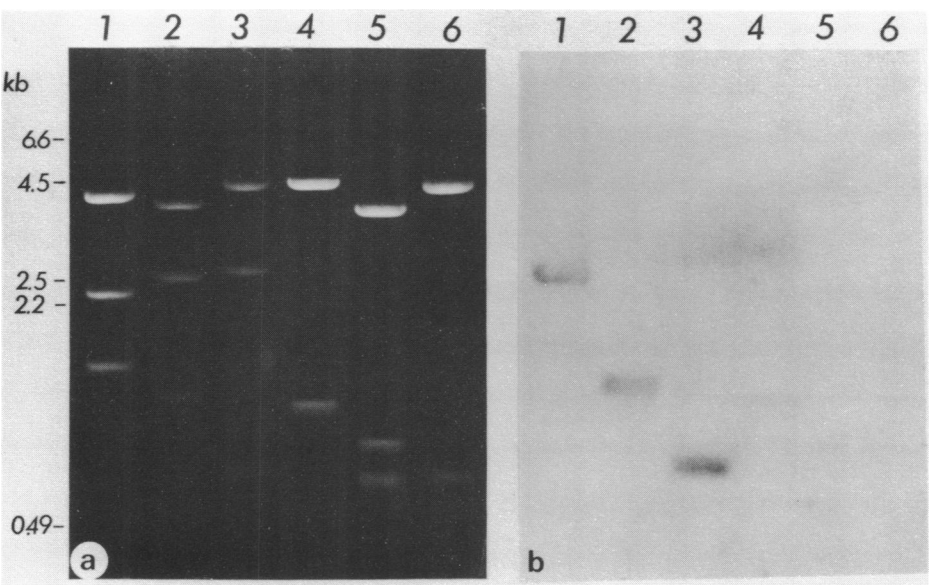

FIGURE 5
Localization of the 5.8S rRNA encoding sequence. (a) Plasmids pMu37 and pMu438 were digested with the appropriate restriction endonucleases and subjected to electrophoresis through a 1.2% agarose gel and stained with ethidium bromide. Lanes 1-3, pMu37 digested with: Lane 1, Hind III and Bam H-1; Lane 2, Hind III and Sal I; Lane 3, Hind III and EcoRI; Lanes 4-6, pMu438 digested with: Lane 4, Hind III; Lane 5, Hind III and Pst I; Lane 6, Hind III and Eco RI. (b) Autoradiograph of the pMu37 and pMu438 restriction fragments hybridized with ^{32}P-labeled 5.8S rRNA.

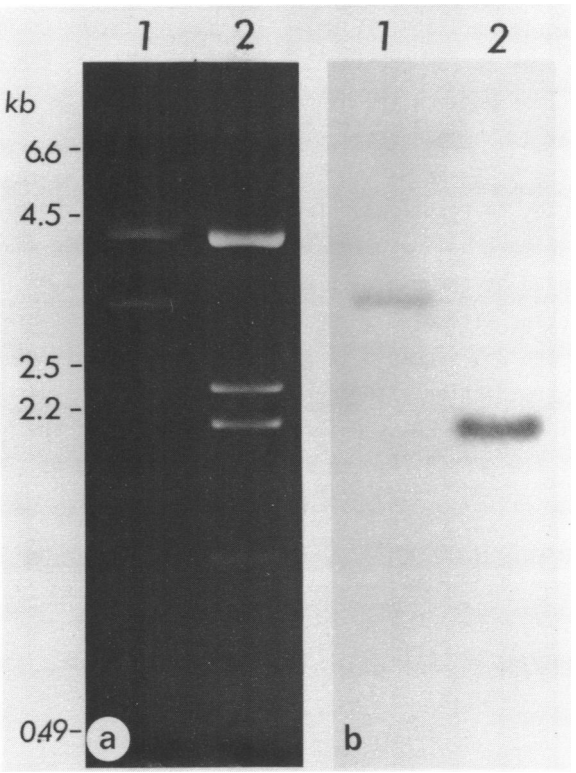

FIGURE 6
Localization of the 5S rRNA encoding sequence. (a) Lane 1, pMu294 digested with Hind III and Bam H-1 (see Fig. 3, Lane 3); Lane 2, pMu300 digested with Hind III and Bam H-1. After digestion fragments were subjected to electrophoresis through 1.2% agarose and stained with ethidium bromide. (b) Autoradigraph of the pMu294 and pMu300 restriction fragments after hybridization to ^{32}P-labeled 5S rRNA.

was, in fact, associated with the basic rDNA repeat. ^{32}P-labeled 5S RNA hybridized to two Hind III, Bam H-1 restriction fragments located between 0 and 1.2 megadaltons as well as between 4.2 and 6.4 megadaltons. The fact that the 18S sequence and 25S sequence extended into the respective fragments suggested the 5S encoding region resides at or near the 0 position and 6.4 position. The results can be explained if: (1) There are two independent 5S sequences, one in the pMu300 insert and one in the pMu294 insert. (2) There is only one 5S sequence which overlaps the 0 and 6.4 positions as

part of the repetitive unit coding for all the rRNA species. Studies are in progress to determine the correct explanation.

DISCUSSION

In order to begin investigations of the organization of the Mucor racemosus genome, recombinant plasmids that contain the rRNA genes of the organism were isolated and characterized. We have determined that the entire rDNA repeat unit is contained on two Bam H-1 restriction fragments with a total molecular weight of approximately 6.4 megadaltons. In addition, sequences that specify all four rRNA species are clustered within this region.

From the results of this and other investigations a clear picture is emerging concerning the overall features of the rDNA organization in eukaryotes. In general, the 18S - 5.8S - 25S arrangement has been found to be the rule. However, beyond this most basic similarity, wide differences exist from one organism to another in regard to the molecular details of the structural organization. For example, the size of the basic repeat unit ranges from the relatively small 5.8 megadalton repeat of Saccharomyces cerevisiae (5) to the approximately 30 megadaltons observed in Dictyostelium discoideum (15). In addition, the gene cluster may exist as a tandem repeat as in Neurospora crassa (16) or in a palindromic arrangement as in Physarum polycephalum (17). Intervening sequences have been observed in the 25S gene in Physarum (18), Drosophila species (19,20) and Tetrahymena pigmentosa, strain 6UM (21). However, in the latter case another strain of Tetrahymena, 8ALP, lacks the intervening sequence (21), and in the case of Drosophila the observed intervening sequence is present in only a portion of the rDNA complement within any individual organism (19,20). On the other hand, no intervening sequences have been observed in Saccharomyces rDNA (22). The 5S RNA encoding sequence has been found both linked and unlinked to the genes that specify the other rRNAs. In the case of Saccharomyces (5), Dictyostelium (15,23), and Mucor the 5S gene is associated with the other rRNA genes. However, in Physarum (24), Tetrahymena (25), Xenopus (26), and most higher eukaryotes examined, the 5S rDNA is not linked with the 18S - 5.8S - 25S rDNA repeat unit. In an extreme case the evidence suggests that the 5S rRNA encoding sequences in Neurospora crassa (15) are, in fact, scattered throughout the genome.

Clearly, the diversity observed in the arrangement of the rDNA region suggests that there is considerable variation from organism to organism in the regulation of transcription of the rRNA species and the processing of

the primary transcript. It is hoped that our continuing investigation of the expression of the rRNA genes in Mucor may lend further insight into these mechanisms.

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