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Linear Mitochondrial Deoxyribonucleic Acid from the Yeast *Hansenula mrakii*

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The mitochondrial deoxyribonucleic acid (mtDNA) from a petite-negative yeast, *Hansenula mrakii*, was studied. A linear restriction map was constructed with 11 restriction enzymes. The linearity of the genome was confirmed by direct end labeling of the molecule, followed by restriction analysis. The molecular weight of the DNA was found to be 55,000 base pairs. This is the first linear mtDNA found in yeast species. Using specific gene probes obtained from *Saccharomyces cerevisiae* mtDNA, we have constructed a gene map of *H. mrakii* mtDNA. The arrangement of genes in this linear genome was very different from the circular mtDNA of other known yeasts.

In most organisms, mitochondrial DNA (mtDNA) is a circular double-stranded molecule. Only two ciliates, *Tetrahymena* and *Paramecium*, have been reported to possess a linear mitochondrial genome (4, 14). We have found that the yeast *Hansenula mrakii* also has a linear mtDNA of about 55,000 base pairs (bp). Since this is the first case for yeast species, we have examined the gene organization in this DNA with respect to the circular mtDNA from other yeasts. We have found that the mitochondrial genes of *H. mrakii* have a considerable degree of sequence homology with the circular *Saccharomyces cerevisiae* mtDNA, but that the arrangement of various genes is very different.

MATERIALS AND METHODS

Strains. The strain of *H. mrakii* used was strain NRRL Y-1364. *S. cerevisiae* MH41-7B is a standard strain whose mtDNA has been extensively studied (10).

Four [*rho*] deletion mutants of *S. cerevisiae* have been used for which the molecular and genetic structures of mtDNA are well known (7; M. Wesolowski, A. Algeri, and H. Fukuhara, Curr. Genet., in press). All of them have a very simple structure. Mutant O_I-2 carries a mitochondrial oligomycin resistance marker O_I^r, and its mtDNA has a unique sequence of 2,300 bp. This genome has the structural gene for the adenosine triphosphatase (ATPase) subunit IX and no other known genes. Mutant O_{II}-3 has an mtDNA with a 920-bp-long sequence carrying the genetic marker O_{II}^r (structural gene of the ATPase subunit VI). Mutant C-5 has an mtDNA of 2,000 bp and represents the 3'-terminal region of the mitochondrial large ribosomal ribonucleic acid (21S rRNA) gene. Mutant P-5 carries a mitochondrial marker P^r (resistance to paromomycin) on its mtDNA of about 800 bp. The molecular nature of the P^r marker is not known. These simple [*rho*] mtDNA's were used as hybridization probes as described below.

Preparation of mtDNA. All strains were grown in a medium containing 2% galactose, 1% yeast extract (Difco Laboratories), 1% peptone (Difco), and 0.1% glucose until early stationary phase. Total DNA was extracted from the cell homogenates as described previously (15). mtDNA was separated by isopycnic centrifugation in CsCl-4',6-diamidino-2-phenylindole dihydrochloride (Boehringer Mannheim Corp.) as described by Williamson and Fennel (16). The isolated mtDNA contained no detectable contamination by nuclear DNA and gave restriction patterns identical to those of the DNA isolated from purified deoxyribonuclease-treated mitochondria.

Preparation of mitochondrial 21S rRNA, 15S rRNA, and 4S RNA. Mitochondria were purified from *S. cerevisiae*, and RNA was extracted according to Faye et al. (3). The RNA was fractionated by electrophoresis on a 1% agarose-6 M urea slab gel. 21S, 15S, and 4S RNAs were excised from the gel and repurified by phenol extraction and ethanol precipitation. The RNA was sheared by alkali (0.05 M Na_2CO_3, 60 min, 50°C; 5) and then 5' labeled by [γ-^{32}P]adenosine triphosphate and T4 polynucleotide kinase.

Labeling of DNA. The mtDNA isolated from various [*rho*] mutants was labeled by nick translation according to Rigby et al. (12), using [α-^{32}P]deoxycytidine triphosphate and DNA polymerase I. Some of the *H. mrakii* restriction fragments were also labeled in the same way to determine the contiguous fragments on the map by overlapping hybridization. End labeling of the whole molecule of *H. mrakii* mtDNA was carried out as follows. DNA (2 μg) was incubated with bacterial alkaline phosphatase (2 U) in 200 μl of 2 mM tris(hydroxymethyl)aminomethane buffer, pH 8, at 45°C for 2 h. DNA was repurified by phenol extraction and alcohol precipitation and then incubated with [γ-^{32}P]adenosine triphosphate (50 μCi; 2,000 Ci/mmol) and T4 polynucleotide kinase (1 U) at

37°C for 30 min. The labeled DNA was passed through a 2-ml column of Sephadex G-100 equilibrated with 0.2 M NaCl and precipitated with ethanol.

Restriction enzyme digestion of DNA and hybridization. The mtDNA of *H. mrakii* was digested by various restriction enzymes (Bethesda Research Laboratories) and by combinations of several enzymes. The DNA fragments were displayed on agarose gels by electrophoresis, and their molecular weights were determined against the reference phage lambda fragments. The mtDNA fragments were transferred to a nitrocellulose paper (13) and incubated with various radioactive probes (DNA or RNA, denatured at 100°C for 3 min) in 0.3 M NaCl–0.1 M sodium phosphate (pH 6.2)–1% Sarkosyl for 24 or 48 h at 45°C. The paper was washed in 0.3 M NaCl, rinsed briefly in 10 mM tris(hydroxymethyl)aminomethane (pH 8), dried, and autoradiographed on Kodak NS-2T films.

Melting curves of hybrids. The hybrids formed on the nitrocellulose paper were incubated in 0.3 M NaCl–50% formamide at increasing temperature, and the radioactivity released at each temperature increment was determined (1).

RESULTS

Restriction fragment map of *H. mrakii* mtDNA. The sum of molecular weights of all fragments generated by individual or mixed restriction enzymes gave a constant value of 55,000 ± 1,500 bp, which can be considered as the total molecular weight of *H. mrakii* mtDNA. However, the number of fragments produced in single-enzyme digestions and mixed digestions was not compatible with a circular arrangement of the fragments. When an enzyme cuts a circular DNA into m fragments and another enzyme into n fragments, the double digestion should produce $m + n$ fragments. We systematically found $m + n - 1$, as though all enzymes shared a common site. This is readily explained if the molecule is linear. Head-to-head circular dimers should also give "$m + n - 1$" restriction patterns, but in that case, the junction fragments should appear in hemimolar amounts. This was not the case. Assuming a linearity, it was possible to construct a linear map coherent with all digestion patterns. The map is shown in Fig. 1.

Linearity of *H. mrakii* mtDNA. To determine whether *H. mrakii* mtDNA has two free termini, we tried to label the molecule at the assumed 5′ ends. After dephosphorylation and ^{32}P phosphorylation according to the usual procedures used for 5′ labeling, the DNA was digested by several restriction enzymes. Only specific fragments were found to be labeled (Fig. 2): *Eco*RI, 2.3 and 5.6 kbp; *Hha*I, 4.8 and 7.8 kbp; *Sac*II, 1.8 kbp; and *Ava*I, 1.4 kbp. These fragments corresponded to the terminal fragments indicated in the restriction map of Fig. 1. However, we could not detect any radioactivity in the *Ava*I 1.8-kbp fragment. Possibly an *Ava*I site is present very close to the left terminus, generating a small fragment that could have run out from the gel. The DNA also has been examined by 3′ labeling, using [α-^{32}P]adenosine triphosphate and terminal nucleotide transfer-

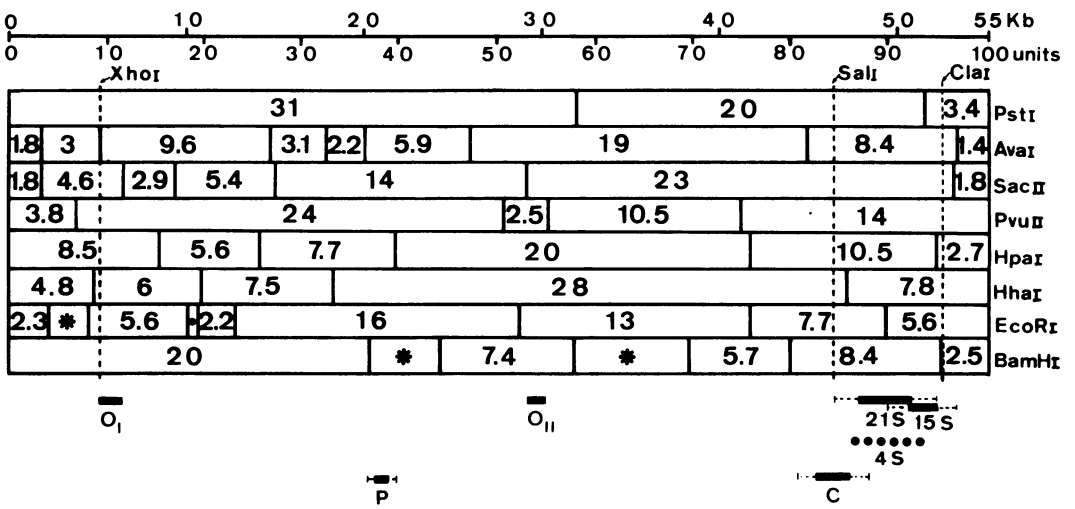

FIG. 1. *Linear restriction map of H. mrakii mtDNA. The numbers indicate the fragment size in base pairs. The asterisks indicate the presence of unordered small fragments: for EcoRI, 1.7, 1.4, and 0.8 kb; for BamHI, 3.0, 2.9, 2.9, and 1.6 kb. Restriction fragments of <500 bp may have escaped detection in this analysis. Dashed lines indicate possible limits of gene position as defined by the nearest restriction sites. The bars placed within the dashed region represent the size of the probe sequence.*

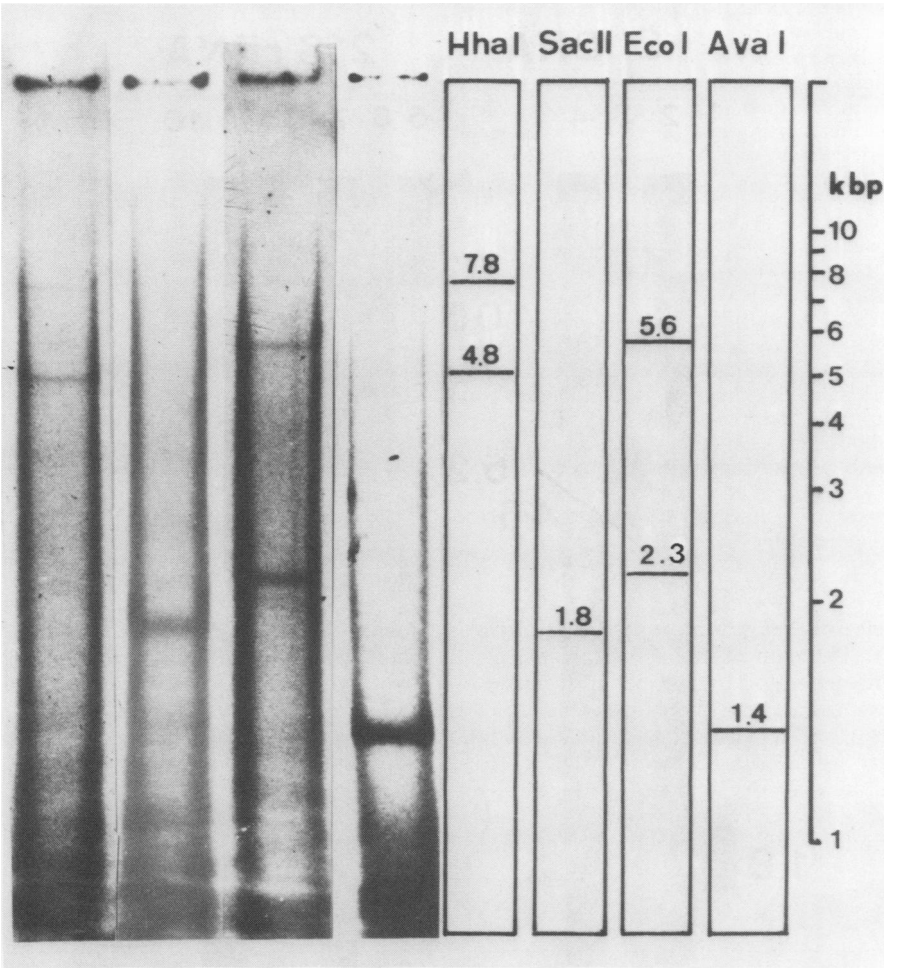

FIG. 2. *5'-End labeling of H. mrakii mtDNA. mtDNA was 5' end labeled as described in the text and digested by restriction enzymes as indicated. After electrophoresis of the digests on an agarose slab gel, the gel was dried on a Gel-bond plastic sheet (Marine Colloid) and autoradiographed.*

ase (data not shown). After restriction by *Eco*RI, *Hha*I, and *Hpa*I, we found the following fragments labeled: *Eco*RI, 2.3 and 5.6 kbp; *Hha*I, 4.8 and 7.8 kbp; and *Hpa*I, 2.7 and 8.5 kbp. This result again confirmed the linear map.

A preparation of *H. mrakii* mtDNA has been examined by electron microscopy. No circular molecules have been found. However, the size of the molecules varied considerably, suggesting a degradation during preparation of the DNA. Therefore, the absence of circles could not be taken as positive evidence for the linearity. Better procedures for preparing this DNA are being developed.

Gene organization of *H. mrakii* mtDNA. In *S. cerevisiae*, a number of mitochondrial genes have been identified and localized on the 75,000-bp mtDNA. These genes can be isolated in the form of [*rho*] deletion genomes, which are, in many cases, a very simple DNA carrying only one single known gene. Such DNA sequences can be used as radioactive probes to detect homologous sequences in the mtDNA from other yeasts and fungi (Wesolowski et al., in press). Radioactive probes for the ATPase subunit genes (O_I^r and O_{II}^r), gene C^r, and gene P^r as well as 21S and 15S rRNA and 4S RNA were prepared and hybridized to the restriction fragments of *H. mrakii* mtDNA. We found that, for all of these probes, defined specific fragments formed radioactive hybrids which were significantly stable (Fig. 3). We determined the half-melting temperatures of these hybrids. In an experiment shown in Fig. 4, the hybrids formed

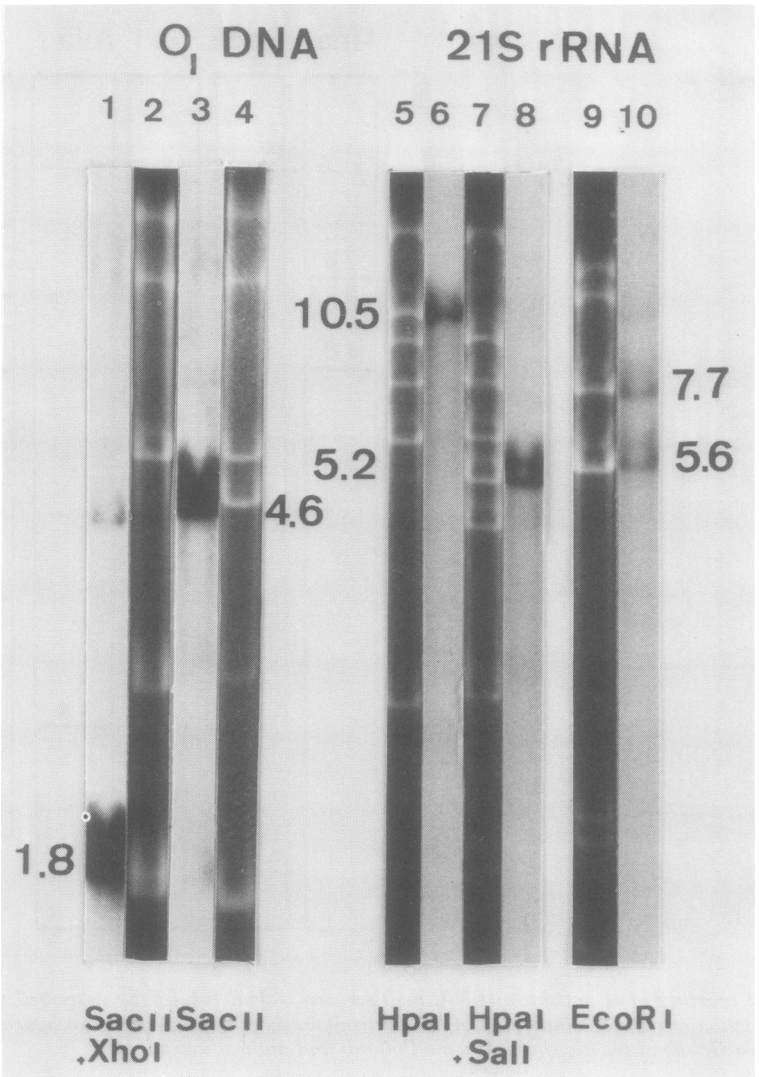

FIG. 3. *Presence of sequences homologous to the mitochondrial genes from S. cerevisiae. Electrophoresis of restriction fragments on agarose gel. DNA fragments were stained with ethidium bromide and photographed under ultraviolet light. The fragments were "Southern transferred" to a nitrocellulose filter and hybridized to radioactive O_I-2 mtDNA (ATPase subunit IX gene) and 21S rRNA. The same nitrocellulose filter was used repeatedly after dehybridization (0.3 M NaCl, 100°C, 5 min). (Lanes 2, 4, 5, 7, 9) Gels stained with ethidium bromide; (lanes 1, 3, 6, 8, 10) autoradiograms. The lengths of the fragments which hybridize are given in kilobase pairs.*

between the O_I probe (ATPase subunit IX) and *H. mrakii* mtDNA had a melting temperature of 37°C (in 50% formamide, 0.3 M NaCl), which was approximately 4°C lower than the homologous hybrids with *S. cerevisiae* mtDNA. The difference corresponds to a mismatch of about 6% (9).

The sequence that hybridized to the ATPase gene has been localized in the genome between map units 9.2 and 11.6. In the same way, the sequences homologous to other probes have all been mapped (see Fig. 1).

DISCUSSION

Since all mtDNA's so far known in yeasts are circular, the finding of a linear mtDNA in *H. mrakii* was surprising. For another *Hansenula*

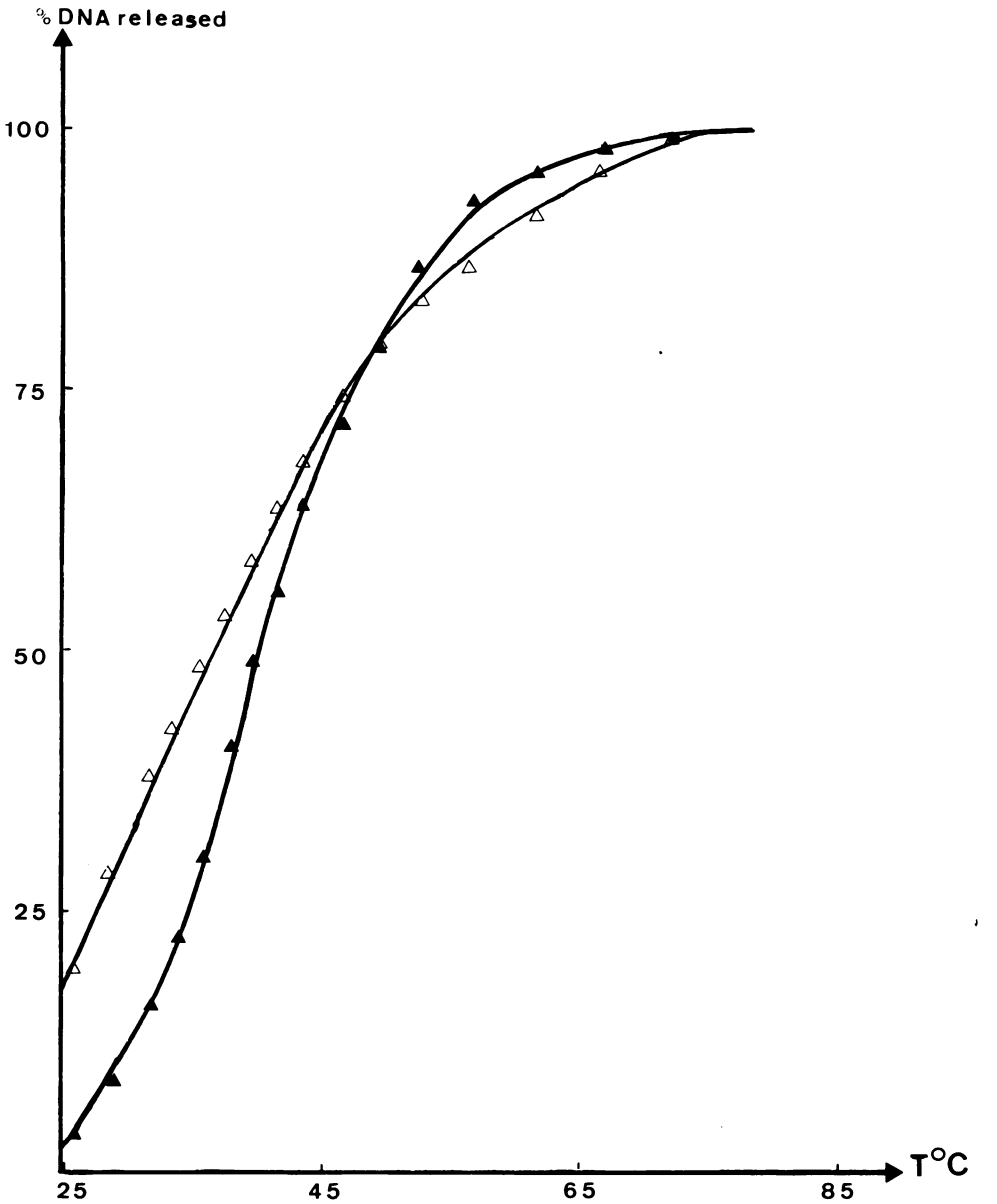

FIG. 4. *Melting curves of hybrids. The labeled mtDNA (5 × 10^5 cpm) from the [rho] O_1-2 strain carrying the ATPase subunit IX gene was hybridized to nitrocellulose-bound mtDNA of H. mrakii (5 μg) or S. cerevisiae (5 μg). The hybrids formed on the filters were incubated at increasing temperatures (see text). The radioactivity released at each increment of temperature was recorded (cumulative plot). Symbols: △, H. mrakii mtDNA; ▲, S. cerevisiae mtDNA.*

species (*Hansenula wingei*), O'Connor et al. (11) have found a circular mtDNA of about 8.2 μm (approximately 25,000 bp).

Among ciliates, *Tetrahymena pyriformis* mtDNA is a linear molecule of about 15 μm (14), and *Paramecium aurelia* mtDNA is also a linear molecule of about 14 μm (4). Goldbach et al. (5) have shown that *Tetrahymena* mtDNA carries a gene for the 21S rRNA near one end of the DNA and a second copy of the gene on the other end, the 15S rRNA gene being in the central region as a single copy. In *H. mrakii* mtDNA, both 21S and 15S rRNA genes are near one extremity of the genome. There is no indication

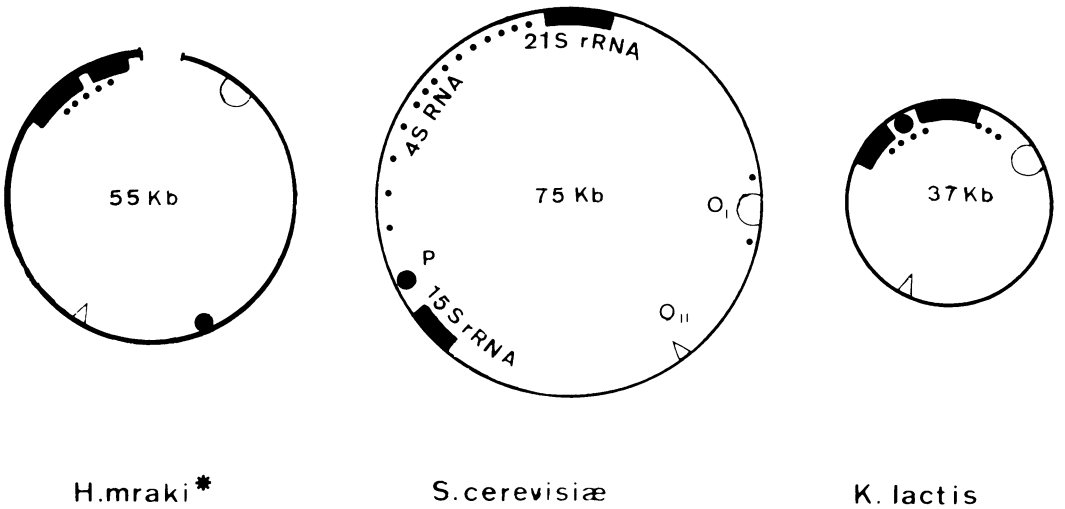

FIG. 5. *Comparison of mitochondrial gene organization in some yeasts. Positions of various genes are schematically indicated. Data for S. cerevisiae and K. lactis were adapted from reference 10 and from Wesolowski et al. (in press). O_I, O_{II}, and P stand for the ATPase subunit IX gene, the ATPase subunit VI gene, and the paromomycin resistance gene, respectively. *The linear H. mrakii is represented as an interrupted circle for the purpose of comparison of genome size.*

of the presence of a duplicated gene on the other extremity of the genome. In *Paramecium* mtDNA, the gene for the large rRNA also has been found near one end of the genome (2).

It has been shown previously that the mtDNA's from yeasts and fungi have a very high degree of sequence homology (8; Wesolowski et al., in press). The map position of rRNA genes in *Kluyveromyces lactis* mtDNA, as determined by *S. cerevisiae* rRNA probes, is in good agreement with the position found by Groot and Van Harten-Loosbroek (6), who used homologous *K. lactis* probes. Therefore, the gene probes available from the *S. cerevisiae* mitochondrial genome are a convenient tool for detection, localization, and isolation of equivalent genes in other fungal species. All of the *S. cerevisiae* probes used here have revealed the presence of homologous sequences in *H. mrakii* mtDNA, although the order of the genes is quite different from the circular yeast mtDNA's (Fig. 5). Our results show that very similar genes can be assembled in highly dissimilar ways to function within the mtDNA's of various sizes and forms found in different yeasts.

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