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THE RIBOSOMAL RNA GENES ON *NEUROSPORA CRASSA* MITOCHONDRIAL DNA ARE ADJACENT

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Summary

Hybridization of separated 24 S and 17 S ribosomal RNA from *Neurospora crassa* mitochondrial ribosomes to restriction fragments of mitochondrial DNA leads to the conclusion that the large and small ribosomal RNA are adjacent on the restriction endonuclease cleavage map of the DNA. The distance between the two genes is estimated at 900 basepairs. This result is consistent with the existence of a ribosomal precursor RNA in *N. crassa* mitochondria and is in contrast to the situation in yeast, where the ribosomal genes are far apart on the mitochondrial DNA. The position of the ribosomal RNA genes on the cleavage map of *N. crassa* mtDNA provides a start for ordering the Hind III restriction fragments.

Introduction

Mitochondrial DNA from *Neurospora crassa* is a double stranded circular molecule with a contour length of 19 μ m, as detected by electron microscopy of osmotically shocked mitochondria [1]. Preparative isolation of mtDNA yields only linear molecules, heterogeneous in size and G + C content [2]. Nevertheless it has been possible to construct a complete cleavage map of the mtDNA with restriction enzymes [3,4]. The sum of the fragment lengths is in excellent agreement with the contour length of the intact molecule indicating

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Abbreviations: mtDNA, mitochondrial DNA, rRNA, ribosomal RNA, 1 $\times$ SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulphate.

that the mtDNA is a collection of homogeneous molecules, without major gene repetition [4].

Previous reports on the hybridization of mitochondrial ribosomal RNA with mtDNA indicated that the DNA contains one gene for the large and one gene for the small rRNA [2,5,6]. Hybridization of the rRNAs to restriction fragments of the mtDNA, described in this article, shows that the genes for the large and small rRNA are located close to each other on the mitochondrial genome. The knowledge of the position of the large rRNA on the cleavage map of the mtDNA has enabled us to extend the map with the order of eight additional Hind III fragments.

The results of our rRNA gene-mapping studies have been presented in a summary form at the Conference on the Genetics and Biogenesis of Chloroplasts and Mitochondria in Munich [7].

Materials and Methods

Isolation of mitochondria and mtDNA from *Neurospora crassa* strain E 5256 has been described [2,4]. Incubation of mtDNA with restriction enzymes and electrophoresis on 0.7% agarose slabgels was done as described previously [4]. Restriction enzymes Eco RI, Hind III and Bam HI were obtained from Miles Laboratories Inc. and from New England Biolabs.

DNA fragments generated by complete digestion with restriction enzymes were transferred to a nitrocellulose stripfilter (Sartorius, 0.45 μm ; 15 $\times$ 0.5 cm) according to Southern [8]. The DNA input per digest was 3 μg .

Mitochondrial rRNA was extracted [9] from 80-S mitochondrial ribosomes [10] and layered on an isokinetic sucrose gradient [11] containing 5–23% sucrose in 30 mM Tris $\cdot$ HCl, 0.1 mM EDTA, 0.2 mM magnesium acetate, pH 7.6 and centrifuged at 27 500 rev./min for 16 h at 4°C in a Beckman SW41 rotor. Fractions sedimenting at 24 S and 17 S were pooled separately and recentrifuged on an identical sucrose gradient. The 24-S and 17-S RNA fractions were precipitated twice with ethanol.

The RNAs were iodinated according to Getz et al. [12] in a final volume of 50 μl . RNA input in the reaction mixture was 20 μg with 0.5–1 mCi of Na^[125]I (New England Nuclear, catalogue No. 033L). The first incubation was at 60°C for 20 min; the second incubation at 70°C for 20 min. The ^[125]I-labelled RNA was purified on a hydroxylapatite (Biorad, DNA grade) column (2 mm $\times$ 7 mm diameter) according to Prenskey et al. [13] and on a Sephadex G-25 column (150 mm $\times$ 7 mm diameter) equilibrated and eluted with 0.1 $\times$ SSC, 0.2% SDS. The excluded volume was precipitated with ethanol together with a 100 fold excess (on a weight basis) of *E. coli* tRNA (Boehringer) and stored at –20°C.

The stripfilters were hybridized to ^[125]I-labelled RNA in a volume of 2.5 ml 3 $\times$ SSC, 0.2% SDS for 19 h at 65°C. Washing of the filters and RNAase treatment was done as described [2]. Stripfilters were subjected to autoradiography (Agfa-Gevaert Curix RP1L) for various times (15 h–5 days). Filter bound radioactivity was counted in toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazolyl)-benzene in the ³H channel of a Mark II scintillation counter (Nuclear Chicago).

Results

The cleavage map of mtDNA obtained with endonucleases Eco RI, Bam HI and Hind III [4] is shown in Fig. 1. For iodination we have isolated 24 S and 17 S rRNA from 80-S mitochondrial ribosomes [9,10]. This rRNA isolated from 73-S mitochondrial ribosomes [2,14], which we consider to be an unfolded conformation of the 80 S ribosome [9,10]. The rRNA from 80-S ribosomes is more intact than the rRNA we extract from 73-S ribosomes [15]. After iodination the rRNA is degraded to fragments of 4 S to 7 S size, but this does not impair the hybridization. Contamination of 80 S rRNA with cytoplasmic rRNA is less than 20% [9,10,14]. Cytoplasmic rRNA does not hybridize with mtDNA [2,14]. To calculate the size of the rRNA genes, we have taken the molecular weight estimates for the 24-S and 17-S rRNAs given by Neupert et al. [16]: $1.28 \cdot 10^6$ and $0.72 \cdot 10^6$, respectively. From these values we calculate a gene size of 3700 basepairs and 2100 basepairs, respectively.

Hybridization of 24 S rRNA to a stripfilter containing Bam HI and Eco RI restriction fragments of mtDNA, reveals that only fragment B1 and E1 are labelled (Fig. 2A and 2B resp.). Hybridization to fragment B1 does not lead to a defined band, due to the large size of B1 (33 000 basepairs) as compared to the mean fragment length of the DNA preparation (15 000 basepairs) [4]. Therefore the majority of DNA fragments to which 24 S rRNA hybridizes, has only one or no Bam HI cleavage site. The hybridization of 24 S rRNA in the region of fragment B2 (Fig. 2A) must be due to these contaminating fragments of B1 since there is no hybridization to E4, E6, E7, E8 and E9 (Fig. 2B) which together contain the whole of B2. These two hybridization experiments localize the 24 S rRNA gene on the overlap of E1 with B1 (Fig. 1). Although the Hind III fragments (Fig. 2C) are not placed on the cleavage map, except for H1, hybridization of 24 S rRNA to Hind III fragments proves to be very infor-

Fig. 1. Restriction fragment map of *Neurospora crassa* mt DNA [4]. Outer segment: Hind III fragment H1. Inner ring: Bam HI fragments. Middle ring: Eco RI fragments are separated with solid lines. The Eco RI fragment sum is 60 550 basepairs ($M_T = 40.3 \cdot 10^6$) [4].

Fig. 2. Hybridization of 24 S mitochondrial rRNA with restriction fragments of mtDNA. A photograph of the gel (a) is aligned with the corresponding autoradiogram of the stripfilter (b). Fragments are numbered in order of decreasing molecular weight. The length of all the fragments can be found in ref. 4. Specific activity of the ^{125}I -labelled RNA is $1.4 \cdot 10^7$ cpm/ μg . RNA input per hybridization: 1.25 μg . (A) Hybridization with a Bam HI digest. (B) Hybridization with an Eco RI digest. (C) Hybridization with a Hind III digest. Due to spillover from a neighbouring lane, the Hind III digest contains about 0.3 μg of an Eco RI digest. The radioactivity present in the band indicated with the broken line can, therefore, be ascribed to hybridization with fragment E1. The labelled Bam HI, Eco RI and Hind III fragments contain respectively 1.8%, 0.6% and 0.7% of the input radioactivity.

mative (Fig. 2C). Fragment H1, H7, H14, H15 and H19 are labelled. Because the 24 S rRNA only hybridizes to E1, this means that fragment H7, H14, H15 and H19 are part of E1 next to H1 (see also Table II). Considering the length of the 24 S rRNA gene (3700 basepairs) and the length of the DNA fragments (Table I) it is clear that of all possible fragment sequences only those sequences are compatible with the hybridization results in which fragment H7 is the fourth fragment following H1. Therefore the fragments H1 and H7 contain the terminal parts of the 24 S rRNA gene and fragment H14, H15 and H19 contain internal parts (Fig. 4). We have also determined whether the position of the 24 S rRNA gene is reflected in the relative amount of RNA hybridizing to each fragment. In theory all internal fragments should bind the same percentage (p) of RNA per unit fragment length (l), if the hybridization conditions are identical for all fragments involved and if DNA loss is as much for all fragments during transfer of the DNA to the filter and during hybridization. That this expectation is reasonably fulfilled, is shown in Table I (fourth column). The p/l ratio is highest for the internal fragments and is nearly the same for fragment H14 and H15. The p/l ratio for fragment H19 is lower and this may be due to preferential loss of small DNA fragments (<500 basepairs) during the transfer of the DNA bands to the filter, as has been reported by Southern [8]. From the per-

Fig. 3. Hybridization of 17 S mitochondrial rRNA with restriction fragments of mtDNA. (A) Hybridization with a Bam HI digest. (B) Hybridization with an Eco RI digest. (C) Hybridization with a Hind III digest. (D) Hybridization with a complete double digest of Hind III plus Bam HI. For hybridization (A), (B) and (C), the input of ^{125}I -labelled RNA is $3\text{ }\mu\text{g}$ per filter with a 50-fold excess of unlabelled 24 S rRNA. The specific activity of the 17 S RNA is $8.5 \cdot 10^6\text{ cpm}/\mu\text{g}$. The labelled Bam HI, Eco RI and Hind III fragments contain respectively 0.06%, 0.125% and 0.05% of the input radioactivity. For hybridization (D) the input of ^{125}I -labelled RNA is $2\text{ }\mu\text{g}$ with a 100-fold excess of unlabelled 24 S rRNA. The specific activity of this 17 S RNA is $4 \cdot 10^6\text{ cpm}/\mu\text{g}$. The labelled bands contain 0.15% of the input radioactivity.

centage of RNA bound to fragment H1 and H7 (Table I, third column) we estimate the overlap of the 24 S rRNA with these fragments at about 450 and 750 basepairs, respectively.

Hybridization of 17 S rRNA was done in the presence of an excess of unlabelled 24 S rRNA to suppress the hybridization of 24-S rRNA fragments contaminating the 17 S rRNA preparation. 17 S rRNA hybridizes to fragment B1, B3, E1 and H1 (Fig. 3A, 3B and 3C). Hybridization of 17 S rRNA with B1 (Fig. 3A) is not visible in the autoradiogram because the radioactivity is spread over a large area (cf. Fig. 2A). It is detectable with scintillation counting of the region involved (Table I). To show in a better way that the 17 S rRNA gene contains a Bam HI recognition site, hybridization was carried out with a strip-filter containing fragments produced in a double digestion of the mtDNA with

TABLE I

QUANTITATION OF STRIPFILTER HYBRIDIZATION

The DNA fragments containing radioactivity from the stripfilters shown in Figs. 2C, 3A and 3D were cut out and counted. Regions of the filter without DNA were used to obtain background values. These were 80, 40 and 25 cpm/mm filterlength in the 3 experiments, respectively and have been subtracted from the areas with labelled bands. A correction was made for residual hybridization of 24 S rRNA, contaminating 17 S rRNA: less than 3% of the total radioactivity bound to the filters in Fig. 3C and 3D was found in bands H7, H14 and H15. The 100% values for the 3 experiments were 12 250, 15 300 and 12 000 cpm (cf. legends to Figs. 2 and 3).

	Fragments hybridizing with RNA	Fragment length (l) (basepairs)	% hybridization (p) 100% = total cpm bound per filter	p/l ($\times 10^3$)
24 S RNA (Fig. 2C)	H1	14 500	10	0.7
	H7	2 470	16	6.5
	H14	1 100	34	31
	H15	980	32	33
	H19	420	8	19
17 S RNA (Fig. 3A)	B1	33 800	25	
	B3	4 650	75	
17 S RNA (Fig. 3D)	HB3	1 750	15	
	B3	4 650	85	

Hind III plus Bam HI (Fig. 3D). Comparison with the Hind III digest (Fig. 3C), shows that five new fragments are present: HB1, HB2, HB3, HB4 and HB5. Fragment H1, H8 and H17 are cleaved by Bam HI because they are not detected in Fig. 3D. As shown previously [4], fragments HB1, HB2 and HB3 are derived from fragment H1 and fragment HB2 is identical to fragment B3 (cf. Fig. 1). Fragment HB3 represents the overlap of B1 with H1 (Fig. 1) and it does hybridize to 17 S rRNA as shown in Fig. 3D. From the percentage of 17 S rRNA binding to B1 and HB3 (Table I), we estimate the overlap of the 17 S rRNA with HB3 at about 400 basepairs. The estimated distance between the large and the small rRNA genes, therefore, is approximately 900 basepairs (Fig. 4).

Digestion of the Eco RI fragment E1 with endonuclease Hind III led to the observation of the fragments listed in Table II. The sum of all the Hind III fragments contained in fragment E1 is in excellent agreement with the expected length of E1, although it remains possible that E1 contains a few more fragments smaller than H19, because of the uncertainty in the calibration of very

Fig. 4. Location of the rRNA genes on Eco RI fragment E1. The bar represents fragment E1 (18 700 basepairs), flanked by fragment E6 and E2 (see also Fig. 1). The hatched regions in E1 indicate the position of the 24 S rRNA gene (3700 basepairs) and the 17 S gene (2100 basepairs). Bam HI recognition sites and Hind III recognition sites are indicated with arrows on the lower and upper side of E1 respectively.

TABLE II

FRAGMENTS OBSERVED AFTER COMPLETE DIGESTION OF E1 WITH ENDONUCLEASE HIND III

Eco RI fragment E1 was extracted from an agarose gel [17], digested with Hind III and electrophoresed in an agarose gel with complete Eco RI and Hind III digests of mtDNA as markers and for calibration [4].

E1 × Hind III fragments	Fragment length (basepairs)
E1H1	6 450
H7a	2 470
H7b	2 470
H10a	1 800
H12	1 300
H13	1 250
H14	1 100
H15	980
E1H2	600
H19	420
Total length	18 890
Exp. length	18 900

TABLE III

COMPOSITION OF PARTIAL FRAGMENTS, OBSERVED IN A DIGEST OF FRAGMENT E1 WITH ENDONUCLEASE HIND III

Fragment E1 was isolated from an agarose gel [17] and partially digested with Hind III. The list of partial fragments was obtained from two different gels, one containing p1–p11 and the other p7–p17. The length of the partial fragments was obtained by calibration with a complete Hind III and Eco RI digest and by interpolation with the terminal fragments of Hind III [4].

Calculated sequence of Hind III fragments, contained in E1				E1H2–7–10–(13,12)–7–19–14–15–E1H1
Partial fragments	Obs. length	Calc. length	Diff. (%)	Composition of partial fragments
p1	18 000	18 290	+1.6	7–10–13–12–7–19–14–15–E1H1
p2	15 500	15 820	+2	10–13–12–7–19–14–15–E1H1
p3	14 000	14 070	+0.5	13–12–7–19–14–15–E1H1
p4	12 900	12 770	–1	12–7–19–14–15–E1H1
p5	11 000	11 470	+4.2	7–19–14–15–E1H1
p6	9 000	9 000	0	19–14–15–E1H1
p7	8 300	8 340	+0.5	10–13–12–7–19–14
p8	7 850	7 520	–4.2	13–12–7–19–14–15
p9	5 950	6 120	+2.0	E1H2–7–10–13
p10	5 150	5 020	+2.5	13–12–7
p11	4 700	4 870	+3.6	E1H2–7–10
p12	4 450	4 350	–2.2	10–13–12
p13	4 200	4 190	–0.2	12–7–19
p14	3 700	3 770	+1.9	12–7
p15	3 300	3 050	–7.5	10–13
p16	1 900	2 080	+9.5	14–15
p17	1 500	1 520	+1.3	19–14

large fragments as E1 [4]. As judged by its fluorescence intensity, band H7, derived from E1, contains two fragments. Together with a fragment of the same size derived from E3 (results not shown), this suggests that the original complete Hind III digest of mtDNA contains three fragments of this size and not two as we had previously reported [4]. Fragment E1H1 (Table II) represents the overlap of E1 and H1 (Fig. 1) [4]. Fragment E1H2 is a new fragment which is also present in a double digestion of mtDNA with Eco RI plus Hind III (results not shown). Therefore we conclude that fragment E1H2 represents the overlap of an unknown Hind III fragment with E1 at the other end of E1. With the list of partial digestion products of E1 with Hind III we have tried to order the Hind III fragments contained in E1 (Table III). This ordering is facilitated by the knowledge of the sequence H7-(H14, H15, H19)-E1H1, deduced from the hybridization of 24 S rRNA to Hind III fragments (Fig. 2C). Since E1 minus E1H1 leaves only 12 450 basepairs, partial fragments p1, p2 and p3 should contain E1H1, located at one side of E1 and can only miss a progressive number of fragments starting from the other end of E1. Therefore, we have looked for the order of (H7, H10, H12, H13) that gives the best fit for these partials and all other partials (Table III). The order of H12 and H13 could not be determined due to the small difference in their lengths (50 basepairs). With this calculation we arrive at the sequence of Hind III fragments given in Table III and Fig. 4.

Discussion

The position of the rRNA genes on *N. crassa* mtDNA (Fig. 4) is fully compatible with the existence of a precursor RNA containing the large and small rRNA [6]. Taking into account the distance between the two genes, we estimate a precursor length of at least 6700 nucleotides. This value is in good agreement with the length estimate of 6900 nucleotides for the ribosomal precursor RNA [6], which was calibrated against the same molecular weights for the rRNAs [16] as used in our calculations.

The location of the rRNA genes in fragment E1 is also in good agreement with the hybridization of the rRNAs to the G + C richer part of the mtDNA molecule [2]. Fragment E1 is relatively rich in G + C, as determined by the denaturation mapping studies of Bernard et al. [18,19]. Comparing the low G + C content of the rRNAs (35% [6]), with the G + C content of E1 (>40% [19]), one would expect to find early melting regions on the denaturation map of E1. Such regions are clearly present in the position expected for the rRNA genes [18,19]. They are flanked by two G + C rich regions and have an G + C rich region in between the two rRNA genes.

The hybridization results, obtained recently by Bernard et al. [20] confirm our result with respect to the hybridization of large plus small rRNA to fragment E1. These authors, however, did not find hybridization to fragment H1. If this is not due to technical difficulties then the mtDNA of strain 7A, used by Bernard et al. [20] should be different from our mtDNA with respect to the Hind III recognition sites, although the Eco RI cleavage maps look identical [3, 4,20].

The arrangement of the rRNA genes in adjacent positions is a strongly con-

served feature for the prokaryotic and eukaryotic genome [21]. Recent data have shown a similar arrangement of the rRNA genes on human [22], *Xenopus* [23] and *Drosophila* [24] mtDNA and also on chloroplast DNA [25]. Reconsidering our own experiments on rat mtDNA [26,27] we conclude now that the rRNA genes on rat mtDNA are also adjacent [28]. An exception to this gene order can be found in yeast mtDNA where the rRNA genes are on opposite parts of the molecule [29–31]. Recently we have found that the region between the two rRNA genes of *N. crassa* mtDNA codes for a 4 S RNA [14]. Such a gene order has previously been reported for animal mtDNA [22,24] and is similar to the gene order in prokaryotic DNA [32]. In eukaryotic nuclear DNA the region between the two rRNA genes contain a 5.8 S gene [21], also in *N. crassa* [33].

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