Volume 3 no.11 November 1976 Nucleic Acids Research

Physical map of Neurospora crassa mitochondrial DNA and its transcription unit for ribosomal RNA

Ulrich Bernard, Claire Goldthwaite and Hans Küntzel

Max-Planck-Institut für Experimentelle Medizin, Abteilung Chemie, Hermann-Rein Strasse 3, 3400 Göttingen, GFR

Received 27 August 1976

ABSTRACT

A circular denaturation and restriction map of mitochondrial DNA from Neurospora crassa is presented. The map shows the position of all twelve fragments produced by restriction endonuclease Eco R I and the position of the largest Hin III fragment along the previously established map of AT-rich sequences. The two wild type strains Em 5256 and 7A differ in the lengths of two Eco R I fragments. No difference was found between the mitochondrial mutant "poky" and its parent strain. The position of the DNA segment carrying the transcription unit for the two ribosomal RNA molecules has been determined by molecular hybridization.

INTRODUCTION

Mitochondrial DNA (mtDNA) from Neurospora crassa is generally isolated as a size-heterogenous mixture of linear molecules, and only a few circular molecules of contour length 20 μm (molecular weight 40 x 10^6) have been detected by electron microscopy [1-3]. The analysis of AT-rich sequences in partially denatured mtDNA has revealed that linear fragments derive from the sequence-homogeneous circular molecule by breaks at randomly distributed positions [3]. Sequence homogeneity was further demonstrated by specific cleavage with restriction endonuclease Eco R I: this enzyme produces 12 specific fragments, and the sum of the fragment lengths is identical with the contour length of the circular molecule [4].

The positions of the four largest Eco R I fragments on the circular denaturation map have been determined by comparing AT-rich sequence patterns[5]. Here we present a complete map of all 12 Eco R I fragments of mtDNA from two different wild type strains of N. crassa obtained by the analysis of partially digested DNA. The DNA region carrying the transcription unit for the two mitochondrial ribosomal RNAs [6,7] was determined by molecular hybridization between specific DNA fragments and in vitro labelled RNA.

MATERIALS AND METHODS

The wild type strains Em 5256 (FGSC 424) and 7A (FGSC 847) and the mitochondrial mutant strain "poky" (FGSC 384) were obtained from the Fungal Genetic Stock Center, Arcata, Calif. Growth of wild type cultures and isolation of mitochondria has been described [8]. The medium for "poky" was supplemented with 200 mg/liter nicotine amide. mtDNA was purified by SDS-phenol extraction and CsCl density gradient centrifugation [9]. Peak fractions of gradients were dialyzed against 150 mM NaCl, 15 mM sodium citrate, 10 mM Tris-HCl pH 8, 1 mM EDTA, pelleted at 150 000 x g and re-dissolved in the above buffer (1:10 diluted) at concentrations of 20 to 30 A_{260}/ml. Radioactive DNA was isolated from cells grown in the presence of 50 µC/l [^{3}H]uracil (Schwartz)[10].

Digestion with restriction endonuclease Eco R I (Miles) was performed in a reaction mixture (100 µl) containing 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 2 µg DNA and 1 µl of enzyme stock solution. The digestion was completed after 20 min incubation at 37°C. For the isolation of partially digested fragments the reaction was stopped after 5 to 12 min incubation by chilling to 0°C and adding 20 µl of a 100 mM EDTA solution.

Digestion with Hin III (Miles) was performed in 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ and 0.1 mg/ml bovine serum albumin at 37°C.

Gel electrophoresis of restriction fragments was performed in 0.5 % agarose (MCI SeaKem) containing 36 mM Tris-HCl pH 7.8, 30 mM sodium phosphate, 1 mM EDTA, for 16 h at 4°, 30 V, in glass tubes of 1 cm diameter. Gels were stained in an aqueous solution of ethidium bromide (0.5 µg/ml) and photographed with Kodak-Panatomic X-film through an orange filter.

DNA was re-isolated from gels using KI gradients [11]. Mitochondrial ribosomes were purified as described [12]. The ribosomal pellet was suspended in a buffer containing 100 mM NH_4Cl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, polyvinyl sulfate (20 µg/ml), heparin (200 µg/ml) and diethyl pyrocarbonate (0.1 %), and the RNA was extracted with SDS-phenol as described [12]. The resulting preparation was dialyzed overnight against the same buffer, treated with DNAse I (Worthington, 5 µg/ml, 37° for 15 min) and then treated with Pronase P (Serva, pre-digested at 37° for 2 h) for 90 min at 37°. The RNA was precipitated with two volumes of ethanol (0.1 M sodium acetate pH 5, overnight at -20°), resuspended in 5 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, dialyzed extensively against the same buffer, again treated with DNAse as above, heated at 80°C for 10 min in the presence of 0.2 % SDS to remove DNAse, precipitated twice more with ethanol, centrifuged at 45 000 rpm for 30 min to remove polysaccharides, ethanol-precipitated again, and the precipitate was dis-

solved in the iodination buffer.

The RNA was iodinated *in vitro* according to Tereba and McCarthy [14] in a reaction mixture (50 µl) containing 7 x 10^{-5} M KI, 100 µC $^{125}I^-$, 1 mM $TlCl_3$, 70 mM sodium acetate pH 4.3 and 40 µg rRNA. After incubation at 70° for 20 min the reaction was stopped by chilling and adding 1 µl of 0.1 M Na_2SO_3 and 15 µl of 1.5 M ammonium acetate buffer pH 9.6. The mixture was heated for 10 min. to 50°, and the labelled RNA was separated from excess iodide by Sephadex G 25 filtration. The [^{125}I]RNA was hybridized with alkali-denatured mtDNA immobilized on nitrocellulose filters as described [6]. Filter-bound radioactivity was counted in Toluol containing 5.5 g Scintimix III/l (Merck) in a Packard Scintillation counter.

RESULTS AND DISCUSSION

Mitochondrial DNA from two different wild type strains of *N. crassa*, 7A and Em 5256, was completely digested with restriction endonuclease Eco R I into 12 specific fragments which were separated by agarose gel electrophoresis (Fig. 1).

The molecular weights of the fragments A to K from strain Em 5256 have already been determined by electron microscopy and by gel electrophoresis in the presence of external standards [4]. The smallest fragment L (m.w. 4 x 10^5) is difficult to visualize and has been overlooked in previous experiments [4,5].

The two wild type strains differ only in two fragments: fragment H' of strain 7A is considerably larger (m.w. 2.5 x 10^6) than fragment H of strain Em 5256 (m.w. 1.7 x 10^6), whereas fragment J' of strain 7A (m.w. 1.25 x 10^6) is somewhat smaller than fragment J of strain Em 5256 (m.w. 1.3 x 10^6). We have also analyzed the Eco R I fragments of mtDNA from the mitochondrial mutant "poky" [15]: the mutant pattern was identical with that of the parent strain 7A (see Fig. 1A).

The positions of the four largest Eco R I fragments (A to D) on the circular denaturation map have already been determined by denaturation mapping of individual fragments [5]. The positions of the smaller fragments could not be determined by this method because their denaturation patterns were not sufficiently characteristic.

In order to obtain a complete map of all fragments we have analyzed additional fragments obtained by limited digestion of mtDNA with Eco R I. A typical agarose gel pattern of partially digested mtDNA containing eleven minor bands in addition to the twelve terminal fragments is schematically presented in Fig. 1B.

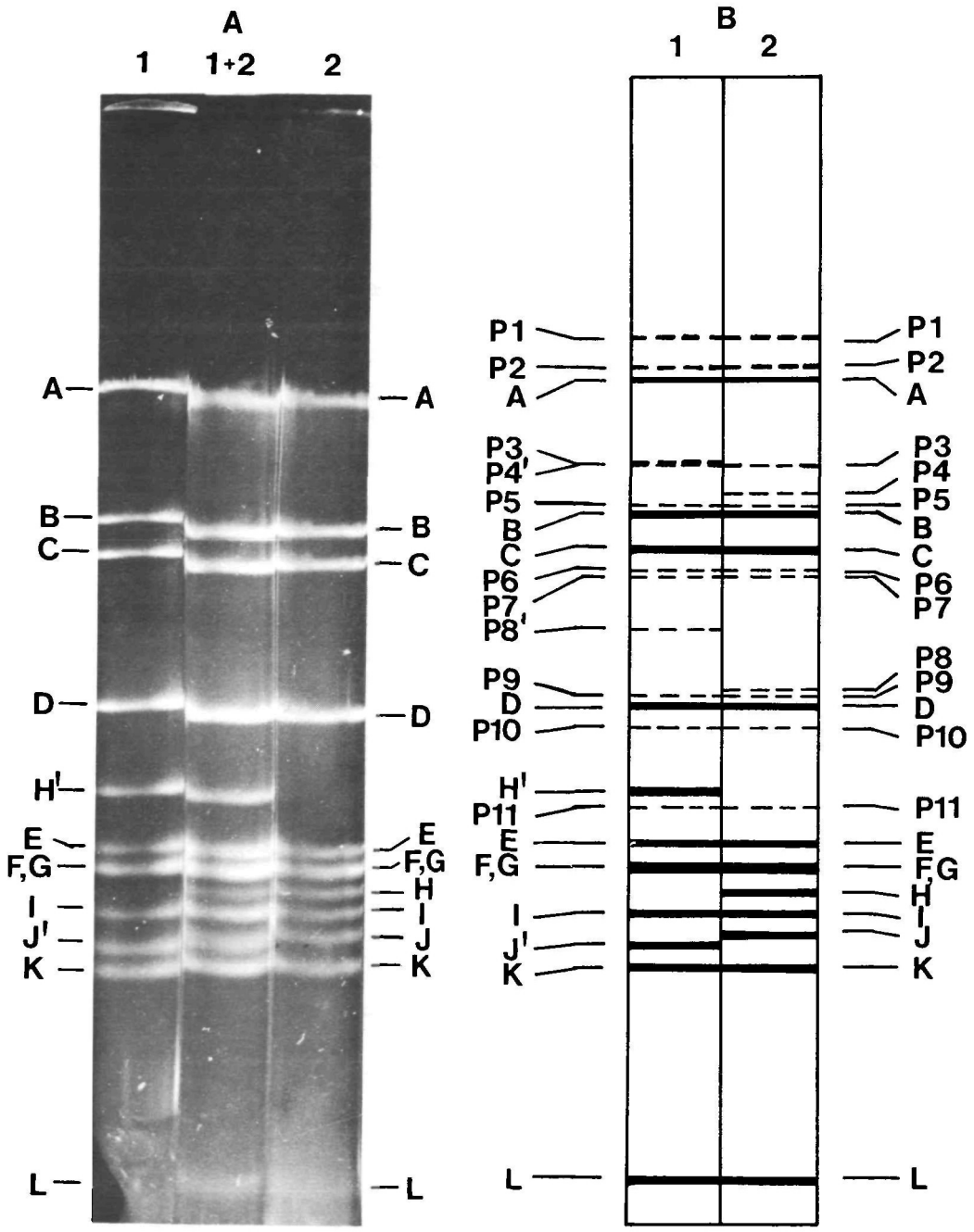

Fig. 1. Agarose gel electrophoresis of Eco R I fragments of mtDNA from N. crassa strains 7A (1) and Em 5256 (2). A, fully digested DNA. B, partially digested DNA.

Table 1. Re-digestion of partially digested Eco R I fragments.

Partially digested fragments	apparent m.w. x 10^{-6}	fragments produced by re-digestion
P 1	21.0	A + B
P 2	16.4	A + E
P 3	9.4	B + K
P 4	8.2	C + H
P 4'	9.4	C + H'
P 5	7.9	C + K
P 6	5.3	D + E
P 7	5.1	D + [F or G]

The partially digested fragments P 1 to P 7 were re-isolated from agarose gels, fully digested with Eco R I and analyzed again by agarose electrophoresis.

The results are summarized in Table 1: All seven partially digested fragments turned out to be pairs of neighboring terminal fragments, and the results of the re-digestion experiments agree well with the apparent molecular weights of the partially digested bands determined by their relative mobility in agarose gels. The data of Table 1 allow us to establish the sequence G(F) - D - E - A - B - K - C - H. Parts of this sequence have already been determined by two other independent methods, namely by re-digestion of the largest Hin III fragment with Eco R I (sequence D - E - A) and by denaturation mapping (sequence D - X - A - B - X - C) [5]. The sequence B - H - C which we had tentatively suggested by using indirect arguments [5] has now been corrected to B - K - C. The partially digested bands P 8 to P 11 could not be isolated in sufficient amounts to allow an analysis by re-digestion. However, from their apparent molecular weights they can be identified as pairs of the terminal fragments indicated in Table 2. The data established the two sequences H - F(G) - L and J - I - G(F). Since F and G cannot be distinguished by their molecular weigths the polarity of the sequence F(G) - L - J - I-G(F) between H and D has to remain open.

The same Eco R I fragments have independently been mapped by Terpstra et al. [17], and their data are in good agreement with ours [16,17].

mtDNA from N. crassa has previously been shown to contain a single set of genes coding for the two mitochondrial rRNAs [6] which are transcribed into a common precursor RNA of molecular weight 2.4 x 10^6 (ref. 7). In order to

Table 2. Identification of partially digested Eco R I fragments as pairs of terminal fragments by their molecular weigths.

Partially digested fragments (m.w. x 10^{-6})	Terminal fragments (m.w. x 10^{-6})
P 8 (3.6)	F or G + H (2.0 + 1.7)
P 8' (4.6)	F or G + H' (2.0 + 2.5)
P 9 (3.5)	F or G + I (2.0 + 1.5)
P 10 (3.1)	I + J (1.5 + 1.3)
P 11 (2.4)	F or G + L (2.0 + 0.4)

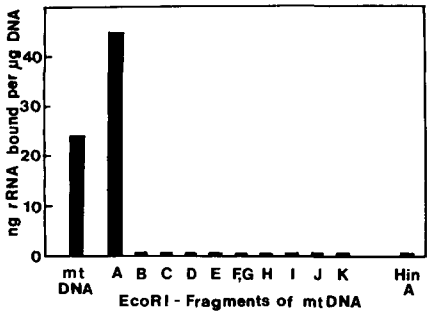

Fig. 2. Hybridization of [^{125}I]-labelled mitochondrial rRNA with 12 restriction fragments and undigested mtDNA. Filters containing 0.2 to 0.3 µg alkali-denatured [^{3}H]-labelled DNA were hybridized with 2 µg RNA (30 to 80 x 10^{3} cpm/µg) as described [6]. The data are mean values of four different experiments. Radioactivity bound to blank filters was substracted from all data. Less than 10 % of filter-bound [^{3}H]-DNA was lost during incubation of filters in the absence of rRNA.

localize the mtDNA region carrying the ribosomal transcription unit we have hybridized all Eco R I fragments (except fragment L) and the largest Hin III fragment A with mitochondrial rRNA. The RNA was extracted with phenol-SDS from purified mitochondrial ribosomes, radioactively labelled *in vitro* by [^{125}I]-iodination and hybridized with alkali-denatured, filter-immobilized DNA fragments. Fig. 2 clearly demonstrates that mitochondrial rRNA hybridizes exclusively with Eco R I fragment A.

The Hin III fragment A which overlaps with about 1/3 of Eco R I fragment A showed no hybridization, suggesting that the ribosomal transcription unit maps within a DNA region of molecular weight 9 x 10^{6} between Eco R I fragment B and Hin III fragment A. This region is only 2 x larger than the transcrip-

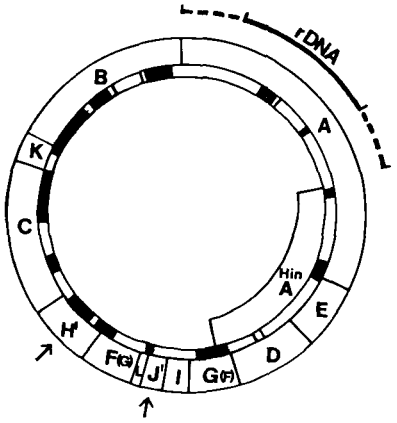

Fig. 3. Physical map of mtDNA from N. crassa (strain 7A). The black regions represent AT-rich segments which are single-stranded in more than 50 % of all analyzed molecules after heating at 49°. The outside letters represent Eco R I fragments. The fragments indicated by arrows have altered m.w. in the strain Em 5256. rDNA: transcription unit for ribosomal RNA.

tion unit for the 32 S ribosomal precursor RNA[7].

The data are summarized in Fig. 3 which shows the circular arrangement of restriction fragments and AT-rich sequences.

The DNA region carrying the transcription unit for rRNA contains long GC-rich sequences which are separated by two short AT-rich segments. Since mitochondrial rRNA has a low GC content (<38 %) [18,7] one has to assume that its transcription unit overlaps with the two AT-rich stretches.

The arrangement of the two cistrons for the large and small rRNA within this unit has not yet been determined. However, it is already clear from these results that the two cistrons are much less separated from each other than in the case of yeast mtDNA [19].

ACKNOWLEDGEMENTS

This study was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 Clayton, D.A. and Brambl, R.M. (1972) Biochem.Biophys.Res.Commun. 46, 1477-1482
2 Agsteribbe, E., Kroon, A.M. and Van Bruggen, E.F.J. (1972) Biochim.Biophys. Acta 269, 299-303
3 Bernard, U., Pühler, A., Mayer, F. and Küntzel, H. (1975) Biochim.Biophys. Acta 402, 270-278
4 Bernard, U., Bade, E. and Küntzel, H. (1975) Biochem.Biophys.Res.Commun.

64, 783-789
5 Bernard, U., Pühler, A. and Küntzel, H. (1975) FEBS-Lett. 60, 119-121
6 Schäfer, K.P. and Küntzel, H. (1972) Biochem.Biophys.Res.Commun. 46, 1312-1319
7 Kuriyama, Y. and Luck, D.J.L. (1973) J.Mol.Biol. 73, 425-437
8 Schäfer, K.P., Bugge, G., Grandi, M. and Küntzel, H. (1971) Eur.J.Biochem. 21, 478-488
9 Nass, M.M.K. (1969) J.Mol.Biol. 42, 529-545
10 Chakraborty, K.P. and Loring, H.S. (1966) J.Biol.Chem. 235, 2122-2126
11 Blin, N., v.Gabain, A. and Bujard, H. (1975) FEBS-Lett. 53, 84-86
12 Datema, R., Agsteribbe, E. and Kroon, A.M. (1974) Biochim.Biophys.Acta 335, 386-395
13 Lizardi, P.M. and Luck, D.J.L. (1971) Nature New Biol. 229, 140-142
14 Tereba, A. and McCarthy, B.J. (1973) Biochemistry 12, 4675-4679
15 Mitchell, M.B. and Mitchell, M.K. (1952) Proc.Nat.Acad.Sci. USA 38, 442-446
16 Bernard, U. and Küntzel, H. (1976) in The Genetic Function of Mitochondrial DNA (Saccone, C. and Kroon, A.M., Eds.) pp. 105-109. North-Holland, Amsterdam
17 Terpstra, P., Holtrop, M. and Kroon, A.M. (1976) in The Genetic Function of Mitochondrial DNA (Saccone, C. and Kroon, A.M., Eds.) pp. 111-118. North-Holland, Amsterdam
18 Küntzel, H. and Noll, H. (1967) Nature 215, 1340-1345
19 Sanders, J.P.M., Heyting, C. and Borst, P. (1975) Biochem.Biophys.Res. Commun. 65, 699-707