Volume 5 Number 2 February 1978 Nucleic Acids Research

Restriction endonuclease cleavage map of mitochondrial DNA from Aspergillus nidulans

Piotr P. Stępień, Ulrich Bernard[1], Howard J. Cooke[2] and Hans Küntzel

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

Received 21 October 1977

ABSTRACT

Mitochondrial DNA of the ascomycete fungus Aspergillus nidulans, a circular molecule of 31 500 base pairs, is cleaved by restriction endonucleases Eco R I, Hind II, Hind III and Bgl II into 3,7,9 and 5 fragments, respectively. The relative positions of the cleavage sites could be mapped by analysis of fragments obtained by double enzyme digestions of whole DNA and by complete and partial redigestion of isolated restriction fragments.

INTRODUCTION

The genetic function of mitochondrial DNA (mtDNA) is currently being studied almost exclusively in the yeast Saccharomyces cerevisiae[1]. However, this otherwise very useful organism is an atypical eukaryote which can exist with highly deleted or even without mitochondrial DNA under anaerobic growth conditions[1]. Only a few of the typical eukaryotes, which appear to depend on their intact mitochondrial genome and their mitochondrial respiratory functions, are amenable to genetic analysis of mtDNA, among them the ascomycete fungi Neurospora crassa and Aspergillus nidulans. The latter seems to be the only organism, except yeast, for which several well separated mitochondrial genetic markers (oligomycin resistance[2], chloramphenicol resistance[3] and cold sensitivity[4]) have been described. This makes A. nidulans a most suitable system to study mitochondrial genes and gene products obligatory for cellular function.

Another interesting feature of A. nidulans is the relatively small size of its mitochondrial genome, a circular DNA molecule of molecular weight 21×10^6 or 31 500 base pairs[5,6]. This DNA is still two-fold larger than metazoan mtDNA[1] but considerably smaller than the mitochondrial genome of the related ascomycete N. crassa (m.w. 40×10^6 or 60 000 base pairs[7,8,9]). A careful analysis of mtDNA and its gene products in these two fungi is needed to answer the intriguing question whether the 1:2 size difference between the

two mitochondrial genomes reflects a different number of structural genes, or merely a different amount of "spacer" or regulatory sequences.

The mitochondrial genome of N. crassa has been studied in two laboratories, using electron microscopy[9,10], restriction endonucleases[6,11-13] and molecular hybridization[14,15]. Here we report a restriction cleavage map of A. nidulans mtDNA.

MATERIALS AND METHODS

Preparation of DNA

All experiments were performed with Aspergillus nidulans strain paba A1, bi A1 (obtained from the collection of the Department of Genetics, Warsaw University). A heavy conidial suspension was added to a minimal medium[15] supplemented with p-amino-benzoic acid (4 µg/ml) and biotine (18 µg/ml), and incubated in an orbital shaker at 37° for 18 to 22 hrs. Mycelium was collected by filtration and washed with cold MTE buffer (0.5 M mannitol, 10 mM EDTA, 30 mM Tris-HCl pH 7.5). All subsequent steps were performed at 4°.

Mycelial pads were homogenized in MTE buffer in a Waring blendor for 30 seconds, and the slurry was passed through a Carborundum mill. The homogenate was centrifuged for 10 min at 4 000 x g, the supernatant was carefully collected, filtered through Miracloth and centrifuged for 10 min at 30 000 x g. Crude mitochondrial pellets were suspended in a small volume of MTE and immediately transferred to lysis buffer (8 M urea, 0.24 M sodium phosphate pH 6.8, 10 mM EDTA, 1 % SDS). Care was taken to perform all steps within one hour or less.

DNA was purified as described by Markov and Ivanov[16], with the following modifications: the mitochondrial lysate was incubated at 55°C for 30 min, cooled and mixed with an equal volume of phenol : chloroform : isoamyl alcohol (50 : 50 : 1, v/v). After 3 min of gentle shaking the mixture was centrifuged for 10 min at 5 000 x g, and solid $NaClO_4$ was added to the water phase to a concentration of 0.8 M. A 4 cm (diameter) x 2 cm (height) column of hydroxyl-apatit (DNA grade, BioRad) equilibrated with 8 M urea, 0.24 M sodium phosphate pH 6.8, 10 mM EDTA was used for chromatography. DNA was eluted with 0.5 M sodium phosphate pH 7.5, the eluate was adjusted to 10 mM EDTA and centrifuged over night in a Beckman Type 50 Ti rotor at 45 000 rpm. DNA pellets were dissolved by gently shaking with 0.2 ml water for one hour.

In most cases DNA obtained by this method was free of nuclear DNA and partially degraded. For some preparations it was necessary to further purify DNA by CsCl density gradient centrifugation[5], and/or to remove small fragments

by centrifugation in a 5 to 20 % sucrose gradient containing 0.1 x SSC for 5 hours at 39 000 rpm in a SW 40 rotor. The various DNA fractions were routinely assayed by cleavage with Eco R I (see below).

Digestion of DNA by restriction endonucleases

Restriction endonucleases Eco R I, Hind II and Hind III were products of Boehringer-Mannheim (Germany), Bgl II was a kind gift of Mrs. Barbara Smith (Edinburgh). Reactions were carried out in volumes of 25 to 100 µl, containing 0.2 to 1 µg DNA, at 37° for 30 min. The reaction mixtures contained 50 mM NaCl, 10 mM $MgCl_2$ (for Bgl II 20 mM $MgCl_2$), 10 mM (Hind II, III) or 100 mM (Eco R I, Bgl II) Tris-HCl pH 7.5. The Hind II mixture also contained 15 mM dithiothreitol.

Gel electrophoresis and re-isolation of DNA fragments

Slab gels (0.5 x 14 x 20 cm) contained 0.6 % or 0.8 % agarose in 0.04 M Tris-acetate pH 8.2, 0.02 M sodium acetate, 1 mM EDTA. Samples were adjusted to 5 % sucrose, 0.5 % SDS and 0.05 % Orange G, and electrophoresis was performed in 4 liters of the above buffer at 2 V/cm for 18 hours at 4°. Gels were stained by soaking in 1 µg/ml ethidium bromide solution and photographed under UV light using Kodak Panatomic X films and orange filter.

DNA fragments recovered from agarose gels according to Blin et al.[17] could be cleaved with Eco R I and Hind III but were completely resistant to Bgl II. Other methods[18,19] were found to be even less satisfactory. For this reason we have developed an alternative procedure based on hydroxylapatite chromatography.

50 µl of Sephadex G-25 (coarse) equilibrated with saturated KI solution was poured into a 1 ml disposable syringe (stoppered by glass wool) and overlaid by 150 µl of hydroxylapatite suspended in saturated KI.

An agarose block (ca. 0.5 g wet weight) containing a DNA fragment (which had been visualized under UV light after soaking in 1 µg/ml ethidium bromide for 1 min) was cut, dissolved in 5 ml of 10 mM sodium phosphate pH 6.8, 1 mM EDTA, saturated KI, and adsorbed to the hydroxylapatite column. The column was washed with 2 ml of saturated KI, followed by 2 ml of 10 mM sodium phosphate pH 6.8, 1 mM EDTA. DNA was eluted with 5 ml of 0.5 M sodium phosphate pH 6.8, the eluate was adjusted to 1 mM EDTA and centrifuged over night at 45 000 rpm, 10°, in a Beckman 50 Ti rotor, and the DNA pellet was dissolved in 100 µl water by 1 hour of reciprocal shaking in the cold.

The restriction digests of these DNA preparations (recovery about 60 %) always contained small amounts of partially digested bands, which were

helpful for mapping. This incomplete digestion is probably not due to absorbed ethidium bromide, since complete removal of the dye did not alter the fragment patterns. When this work was completed, a similar method for recovery of fragments was published[20].

RESULTS AND DISCUSSION

The electrophoretic patterns of specific fragments obtained by complete digestion with restriction endonucleases[21] are shown in Fig. 1. Endonuclease Eco R I produces three fragments (gels A, H), Hind II seven fragments (gel C), Hind III nine fragments (gels E,F) and Bgl II five fragments (gel J). The Eco R I and Hind III patterns of gels A,H and E,F, respectively, show slight variations because of different agarose concentrations used. Some of the smaller fragments are difficult to visualize after reproduction.

The molecular weights of the fragments were determined by co-electrophoresis with the well-characterized Eco R I and Hind III fragments of lambda DNA[22,23]. In some experiments we also used the previously characterized Eco R I fragments of N. crassa mtDNA[12,13] as internal standards. The molecular weights of the fragments are listed in Table 1.

The molecular weights of each single and double digest add up to a total of 21 x 10^6 (31 500 base pairs) which closely corresponds to the molecular size of a 10 µm circular DNA molecule found in A. nidulans mtDNA by electronmicroscopy[5]. We conclude that A. nidulans mtDNA is as homogenous as that of N. crassa, and we confirm that A. nidulans mtDNA has only half the size of that of N. crassa[7-9].

Double digestion with Eco R I and Hind II produces nine bands (Fig. 1B), the four smallest fragments (bands 6 to 9) comigrating with the Hind II fragments h4, h5, h6 and h7. The other three Hind II fragments and all three Eco R I fragments are replaced by six overlapping sequences labelled Eh 1 to Eh 6 (Table 1). It is obvious that the three Eco R I cuts have split each of the Hind II fragments h1, h2 and h3 into two subfragments. The molecular weights of these subfragments (Table 1) allow only one combination: Eh1 + 2 = h1, Eh4 + 5 = h2, and Eh3 + 6 = h3. The large size of Eh1 immediately identifies it as the overlap between E1 and h1. This was directly confirmed by re-digestion of isolated fragment E1 with Hind II, which produced the fragment Eh 1 together with Eh 5 and Hind II fragments h5, h6 and h7 (Table 2).

Re-digestion of fragment E2 with Hind II produced Hind II fragment h4 and the two overlapping fragments Eh3 and Eh4 (Table 2). It follows that h2 overlaps

Table 1. Molecular weights (x 10^{-6}) of fragments generated by single and double digestions with restriction endonucleases. The numbering of gel bands refers to Fig. 1

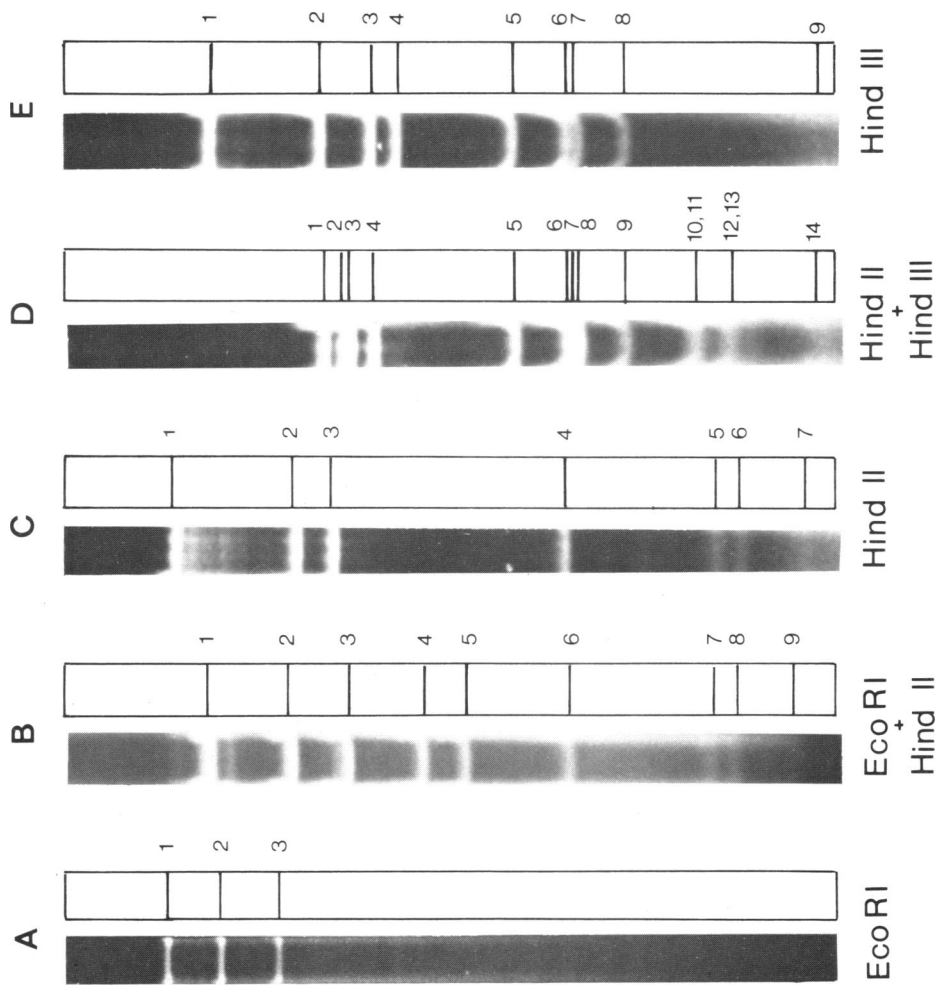
gel band	Eco R I	Hind II	Bgl II	Hind III
1	10.7 E1	11.5 h1	7.8 B1	7.5 H1
2	6.2 E2	3.8 h2	7.2 B2	3.5 H2
3	4.2 E3	3.2 h3	4.2 B3	2.8 H3
4	21.1	1.1 h4	1.0 B4	2.4 H4
5		0.6 h5	0.8 B5	1.3 H5
6		0.5 h6	21.0	1.1 H6
7		0.4 h7		1.0 H7
8		21.1		0.9 H8
9				0.4 H9
				20.9

gel band	Eco R I + Hind II	Eco R I + Bgl II	Eco R I + Hind III	Hind II + Hind III
1	7.5 Eh1	4.3 EB1	6.2 E2	3.5 H2
2	4.0 Eh2	4.2 E3	3.5 H2	3.2 Hh1
3	3.0 Eh3	4.2 B3	2.5 EH1	3.2 h3
4	2.1 Eh4	2.9 EB2	2.4 H4	2.8 H3
5	1.7 Eh5	1.9 EB3	1.3 H5	1.3 H5
6	1.1 h4	1.7 EB4	1.1 H6	1.1 H6
7	0.6 h5	1.0 B4	1.0 H7	1.1 h4
8	0.5 h6	0.8 B5	1.0 EH2	1.0 H7
9	0.4 h7	21.0	0.9 H8	0.9 H8
10	0.2 Eh6		0.8 EH4+H9	0.6 Hh2
11	21,1		0.5 H9	0.6 h5
12			0.4 EH3	0.5 h6
13			0.3 EH4	0.5 Hh3
14			21.9	0.4 h7
15				0.4 H9
				21.1

with E1 by Eh5, and with E2 by Eh4. It further follows that h1 overlaps with E1 by Eh1, and with E3 by Eh2.

These data allow the construction of an unequivocal map of the Hind II fragments relative to the Eco R I fragments (Fig. 2), except that the sequence of h5, h6 and h7 between h1 and h2 remains to be established.

Double digestion with Eco R I and Hind III produces 13 bands (Fig. 1G, Table 1). Bands 3 and 4 of Fig. 1G appear as a single broad band but can be resolved into the two components at low DNA input. The same is true for bands 6,7 and 8, which, at lower DNA input, are resolved into an upper band (component 6) and a lower one with the relative intensity of a double band


A
B
C
D
E
Eco RI
Eco RI + Hind II
Hind II
Hind II + Hind III
Hind III

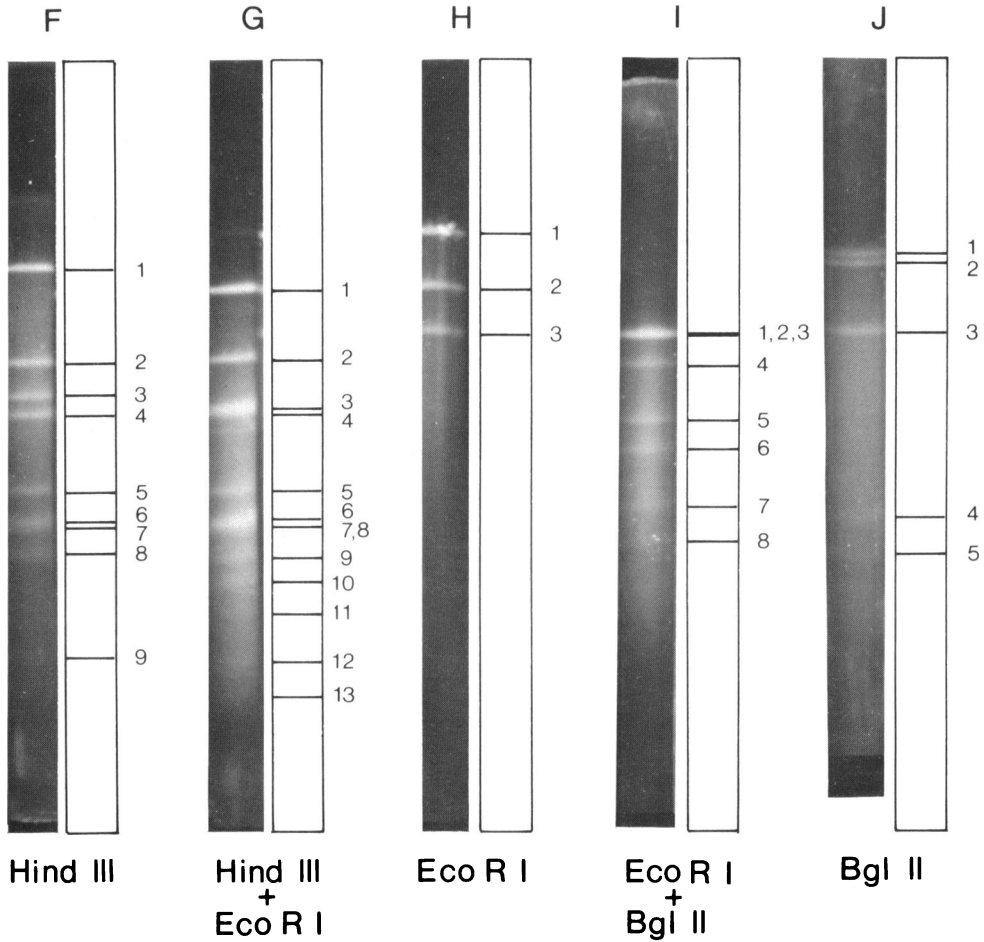


Fig. 1. Agarose gel electrophoresis of A. nidulans mtDNA cleaved by restriction endonucleases. Agarose concentrations were 0.8 % (gels A to E) and 0.6 % (gels F to J).

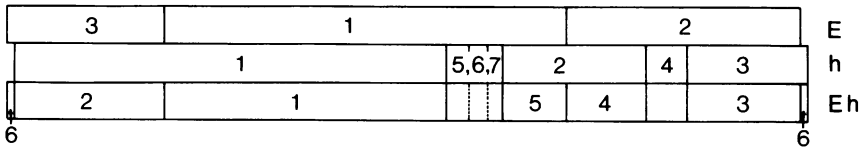


Fig. 2. Colinearity of the Eco R I and Hind II fragment maps.

(components 7 and 8). That the latter band contains two unresolved components has been confirmed by re-digestion experiments (Table 2), which produce two subfragments (EH2 and H7) of the same electrophoretic mobility. The molecular weights of the fragments produced by Eco R I + Hind III double digestion add up to a value (21.9×10^6) which is slightly but significantly higher than that obtained by the other digestions. This is explained by the finding that band 10 is a partially digested fragment containing the pair EH4 and H9.

From the eight bands of the double digest one co-migrates with Eco R I fragment E2, and seven co-migrate with Hind III fragments H2,4,5,6,7,8 and 9. Fragments E1, E3, H1 and H3 are replaced by the four overlapping fragments EH1,2,3 and 4. The subfragment EH1 is produced by re-digestion of E1 with Hind III, and by re-digestion of H3 with Eco R I (Table 2), which identifies it as the overlap between E1 and H3.

Similar re-digestion experiments (Table 2) have shown that EH3 is an overlap between H3 and E3. This places H3 at the junction between E3 and E1.

Re-digestion of H1 with Eco R I produces E2 together with EH2 and EH4. Furthermore, reciprocal re-digestion shows that EH2 is also a subfragment of E1, and EH4 a subfragment of E3 (Table 2). It follows that H1 includes E2 and overlaps with E1 by EH2 and with E3 by EH4. As already mentioned above, a partially digested fragment containing EH4 and H9 has been found in the Eco R I and Hind III double digest. H9, on the other hand hs been shown to be included in E3 together with H6,7 and 8, whereas H2,4 and 5 were found to be subfragments of E1 (Table 2).

These results imply the sequence: H1 - H9 - [H6,7,8] - H3 - [H2,4,5] - H1. We have tried to determine the order of fragments H6,7,8 between H9 and H3 by partial digestion of E3 with Hind III, but the molecular weights of the partially digested fragments did not allow unambiguous selection between the six possible permutations. The sequence of H2,4,5 between H3 and H1, as shown in Fig. 3, could be established by further experiments (see below).

Table 2. Re-digestion of isolated fragments.

E1 + Hind II		E1 + Bgl II		E1 + Hind III		E2 + Hind II		E2 + Bgl II		E3 + Hind III	
7.5	Eh1	4.2	B3	3.5	H2	3.0	Eh3	4.3	EB1	1.1	H6
1.7	Eh5	2.9	EB2	2.5	EH1	2.1	Eh4	1.9	EB3	1.0	H7
0.6	h5	1.7	EB4	2.4	H4	1.1	h4	6.2		0.9	H8
0.5	h6	1.0	B4	1.3	H5	6.2				0.4	H9
0.4	h7	0.8	B5	1.0	EH2					0.4	EH3
10.7		10.6		10.7						0.3	EH4
										4.1	

H1 + Eco R I		H1 + Bgl II		H2 + Bgl II		H3 + Eco R I		H3 + Bgl II	
6.2	E2	5.3	HB1	2.3	HB2	2.5	EH1	2.2	HB3
1.0	EH2	2.1	HB4	1.0	B4	0.4	EH3	0.6	HB6
0.3	EH4	7.4		0.2	HB8	2.9		2.8	
7.5				3.5					

Fig. 4 shows the colinearity of the Hind II and Hind III maps as predicted from the results of Figs. 2 and 3. It should be kept in mind that the order of fragments H2,4 and 5 is still open. According to the arrangement of Fig. 4 the Hind II + Hind III double digest should contain Hind II fragments h3 and h4, and Hind III fragments H3,6,7,8 and 9, whereas fragments h1, h2 and H1 should be missing. This prediction was experimentally verified (Fig. 1D and Table 1). Furthermore, the double digest contains h5, h6, h7, H2 and H5, but not H4. This indicates that H4 is adjacent to H1 and includes h5,6,7, whereas h1 includes all Hind III fragments except H1 and H4.

Fig. 4 also indicates that the Hind II cleavage site between h3 and h1, and the Hind III cleavage site between H1 and H9 have a distance of less than 150 base pairs, since a reduction of fragments H9 and H3 by 0.1×10^6 Daltons (150 base pairs) would have been detected. The remaining ambiguity of the Hind III map, the order of H2 and H5 between H3 and H4, has been resolved by double digestion with Hind III and Bgl II (see below).

Double digestion with Eco R I and Bgl II produces six bands (Fig. 1I). The relative intensity of the first band indicates the presence of three co-migrating fragments. Two of them have been identified as Eco R I fragment E3 and Bgl II fragment B3, because both fragments, when re-isolated from

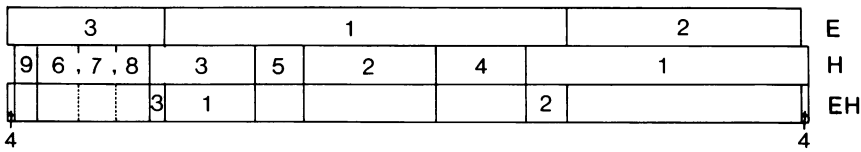


Fig. 3. Colinearity of the Eco R I and Hind III maps.

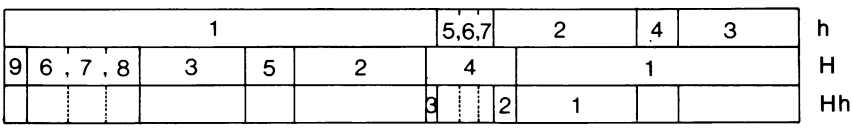


Fig. 4. Colinearity of the Hind II and Hind III maps.

single digests and incubated with the reciprocal enzymes, remained uncleaved. The double digest also contains two bands co-migrating with Bgl II fragments B4 and B5. Two Eco fragments (E1 and E2) and two Bgl fragments (B1 and B2) are replaced by four overlapping sequences (EB1 to 4). EB1 which is formed by re-digestion of E2 with Bgl II (Table 2) co-migrates with E3 and B3 and thus represents the third component of the double digest (Fig. 1I).

From the molecular weights of the overlapping fragments it is obvious that B1 is cleaved by Eco R I into E3, EB3 and EB4, and B2 into EB1 and EB2. No other combinations of these subfragments are possible. Since EB3 and EB4 are found by re-digestion with Bgl II of E2 and E1, respectively (Table 2), it follows that B1 includes E3 and overlaps with E2 by EB3, and with E1 by EB4. The remaining three Bgl fragments B3,4,5 are subfragments of E1 (Table 2). EB1 and EB2 are subfragments of E2 and E1, respectively, and thus represent the overlaps of B2 with E1 and E2.

These data indicate the sequence shown in Fig. 5. The order of B3,4,5 between B1 and B2 could be established by other experiments (see below).

Fig. 6 shows the co-linearity of the Hind III and Bgl II maps as predicted from the previously established sequences (see Figs. 3 and 5). The unambiguous portions of this double map are indicated by heavy lines. This arrangement was confirmed by re-digestion of Hind III fragments H1 and H3 by Bgl II, which produced the subfragments HB1 + HB4 and HB3 + HB6, respectively (Table 2).

Isolated Hind III fragment H2 is split by Bgl II into B4 and the two

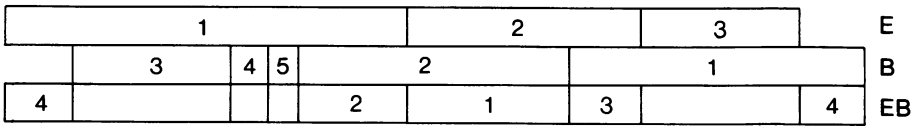


Fig. 5. Colinearity of the Eco R I and Bgl II maps.

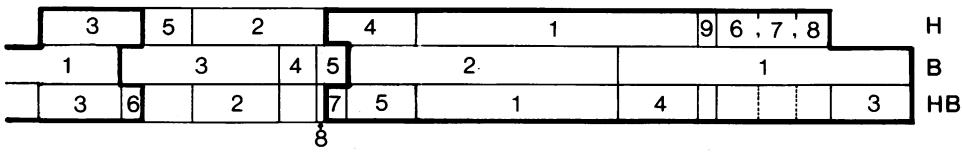


Fig. 6. Colinearity of the Hind III and Bgl II maps.

overlapping sequences HB2 and HB8 (Table 2). There are only two possible arrangements of the fragments between the fixed portions of the map (Fig. 6) which are compatible with this result: the sequence shown in Fig. 6 and in its reversed polarity (H3 - 2 - 5 - 4, B1 - 5 - 4 - 3). In order to discriminate between these two possibilities, we have partially digested isolated fragment E1 with Bgl II. Table 3 shows all possible combinations of final fragments produced by partial digestion, and the fragments found in the partial digest.

It is obvious that two of the observed fragments (molecular weights 6.9 and 3.7 x 10^{-6}) can derive only from sequence (2) (EB4 - B3 - B4 - B5 - EB2), whereas none of the four possible combinations characteristic only for sequence (1) were found. We therefore conclude that the sequence of Bgl II fragments shown in Figs. 5 and 6, and consequently the Hind III sequence of Figs. 3,4 and 6 are correct.

The results are summarized in Fig. 7, showing the complete restriction map of circular A. nidulans mtDNA. The molecule is presented in a linearized form, starting and ending at the junction between Eco R I fragments E1 and E3. The scale represents map units, and one map unit corresponds to a molecular size of 2.1 x 10^{5}, or 315 base pairs. 24 cleavage sites have been mapped, and two of them (at map position 81) appear to coincide. The only remaining ambiguities are the orders of Hind II fragments h5,6,7 and of Hind III fragments H6,7,8, which can be resolved only by using additional restriction endonucleases.

Table 3. Partial digestion of fragment E1 with Bgl II.

Possible sequences EB4 - B5 - B4 - B3 - EB2 (1) EB4 - B3 - B4 - B5 - EB2 (2)	Molecular weight (M_r x10^{-6}) of fragments predicted for sequence (1)	(2)	found in partial digest
[B3 - B4 - B5]- EB2	8.9	8.9	-
[B4 - B3]- EB2	8.1	-	-
EB4 -[B3 - B4 - B5]	7.7	7.7	-
B3 - EB2	7.1	-	-
EB4 - B3 - B4	-	6.9	6.9
[B3 - B4 - B5]	6.0	6.0	6.0
EB4 - B3	-	5.9	-
[B3 - B4]	5.2	5.2	5.2
[B4 - B5]- EB2	-	4.7	-
B3	4.2	4.2	4.2
B5 - EB2	-	3.7	3.7
EB4 - B5 - B4	3.5	-	-
EB2	2.9	2.9	2.9
EB4 - B5	2.5	-	-
[B5 - B4]	1.8	1.8	1.8
EB4	1.7	1.7	1.7
B4	1.0	1.0	1.0
B5	0.8	0.8	0.8

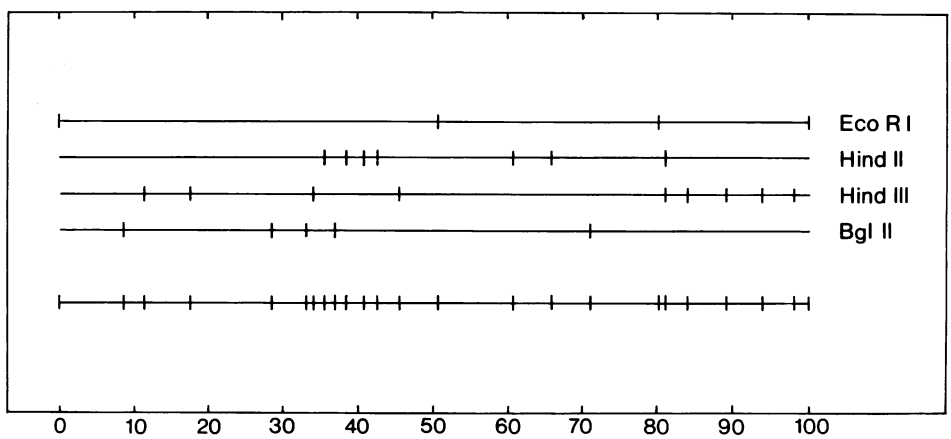


Fig. 7. Relative positions of restriction cleavage sites on A. nidulans mtDNA. The scale represents map units of the linearized molecule.

However, since all these fragments within both groups are of similar small size, the cleavage sites at map positions 37, 38, 89 and 94 would shift maximally by one map unit to the left, assuming some alternative sequences.

All other sites are mapped within $\pm$ 0.1 x 10^6 Daltons or 150 base pairs.

The data reported here provide the experimental basis for further mapping studies: mitochondrial genes for ribosomal and transfer RNAs will be localized by molecular hybridization[12,13], and structural genes for proteins like the mitochondrially synthesized ATPase proteolipid[24] could possibly be identified by programming cell-free systems with restriction fragments[25].

ACKNOWLEDGEMENTS

One of us (P.P.S.) is a fellow of the Alexander von Humboldt foundation. We thank Dr. C. Goldthwaite for advice, and Sigrid Mennicke and Marion Dornwell for technical assistance.

[1]Present address: Department of Biology B-022, University of California San Diego, La Jolla, California 92093, USA

[2]Permanent address: Medical Research Council, Mammalian Genome Unit, Department of Zoology, University of Edinburgh, West Mains Road, Edinburgh, U.K.

REFERENCES

1 Bücher, T., Neupert, W., Sebald, W. and Werner, S. (eds.), Genetics and Biogenesis of Chloroplasts and Mitochondria, North Holland Publ., Amsterdam, 1976.
2 Rowlands, R.T. and Turner, G. (1973) Mol.Gen.Genet. 126, 201-216.
3 Gunatilleke, I.A.U.N., Scazzocchio, C. and Arst, Jr., H.N. (1975) Mol.Gen.Genet. 137, 269-276.
4 Waldron, C. and Roberts, C.F. (1973) J.Gen.Microbiol. 78, 379-381.
5 López Pérez, M.J. and Turner, G. (1975) FEBS-Lett. 58, 159-163.
6 Bernard, U. and Küntzel, H. (1976) in The Genetic Function of Mitochondria (Saccone, C. and Kroon, A.M., eds.) North Holland Publ., Amsterdam, pp. 111-118.
7 Clayton, D.A. and Brambl, R.M. (1972) Biochem.Biophys.Res.Commun. 46, 1477-1482.
8 Agsteribbe, E., Kroon, A.M. and van Bruggen, E.F.J. (1972) Biochim.Biophys. Acta 269, 299-303.
9 Bernard, U., Pühler, A., Mayer, F. and Küntzel, H. (1975) Biochim.Biophys. Acta 402, 270-278.
10 Bernard, U., Pühler, A. and Küntzel, H. (1975) FEBS-Lett. 60, 119-121.
11 Bernard, U., Bade, E. and Küntzel, H. (1975) Biochem.Biophys.Res.Commun. 64, 783-789.
12 Bernard, U., Goldthwaite, C. and Küntzel, H. (1976) Nucleic Acid Res. 3, 3101-3108.
13 Terpstra, P., Holtrop, M. and Kroon, A.M. (1977) Biochim.Biophys. Acta 475, 571-588.
14 Terpstra, P., Holtrop, M. and Kroon, A.M. (1977) Biochim.Biophys. Acta 478, 146-155.
15 Cove, D.J. (1966) Biochim.Biophys. Acta 113, 51-56.
16 Markov, G.G. and Ivanov, I.G. (1974) Anal.Biochem. 59, 555-563.

17 Blin, N., Gabain, A.v. and Bujard, H. (1975) FEBS-Lett. 53, 84-86.
18 Petterson, U., Mulder, C., Delius, H. and Sharp, P.A. (1973) Proc.Nat.Acad. Sci. USA 70, 200-204.
19 Thuring, R.W.J., Sanders, J.P.M. and Borst, P. (1975) Anal.Biochem. 66, 213-220.
20 Moore, K.H., Johnson, P.H., Chandler, S.E.W. and Grossman, L.I. (1977) Nucleic Acid Res. 4, 1273-1289.
21 Roberts, R.J. (1976) Critical Rev.Biochem. 4, 123-164.
22 Thomas, M. and Davis, R.W. (1975) J.Mol.Biol. 91, 315-328.
23 Wellauer, P.K., Reeder, R.H., Carrol, D., Brown, D.D., Deutch, A., Higashinakagawa, T. and David, I.B. (1974) Proc.Nat.Acad.Sci. USA 71, 2823-2837.
24 Mahariel, M.A., Imam, G., Nelson, P., Stępień, P.P. and Küntzel, H. (1977) Eur.J.Biochem. 76, 345-354.
25 Küntzel, H. and Blossey, H.C. (1974) Eur.J.Biochem. 47, 165-171.