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Published in:
Biochimica et Biophysica Acta %28BBA%29 - Nucleic Acids and Protein Synthesis

DOI:
10.1016/0005-2787(77)90318-5

Publication date:
1977

Biochimica et Biophysica Acta, 475 (1977) 571—588

BBA 98920

A COMPLETE CLEAVAGE MAP OF *NEUROSPORA CRASSA* mtDNA OBTAINED WITH ENDONUCLEASES Eco RI AND Bam HI

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(Received November 17th, 1976)

Summary

A physical map of *Neurospora crassa* mitochondrial DNA has been constructed using specific fragments obtained with restriction endonucleases. The DNA has 5 cleavage sites for endonuclease Bam HI, 12 for endonuclease Eco RI and more than 30 for endonuclease Hind III. The sequence of the Eco RI and Bam HI fragments has been established by analysis of partial fragments. By digestion of the Eco RI fragments with Bam HI, a complete overlapping map has been constructed. The position of the largest Hind III fragment on this map has also been determined. The map is circular and the added molecular weight of the fragments is $40 \cdot 10^6$, which is in good agreement with earlier measurements on intact DNA, using the electron microscope.

Introduction

The mitochondria of *Neurospora crassa* contain 19—20 μm duplex circular DNA [1—3]. On this DNA there is one gene for each of the mitochondrial rRNAs [4—6] and a number of tRNA genes [7]. The exact number of tRNA genes remains to be established, but is probably not much higher than 30 (H. de Vries and A. Shtal, personal communication). Further, it has been demonstrated that the mitochondrial ribosomes take part in the synthesis of some mitochondrial inner membrane proteins: 3 from the 7 subunits of cytochrome oxidase [8], 2 of the 9 subunits of the ATPase complex [9] and one of the 7 subunits of the cytochrome bc_1 complex [10]. Although it is very likely that the genes for these proteins are located on the mtDNA, direct

Abbreviations: Eco RI, restriction endonuclease from *Escherichia coli* RY13; Bam HI, restriction endonuclease from *Bacillus amyloliquefaciens* H; Hind III, restriction endonuclease from *Haemophilus influenzae* Rd; Hae III, restriction endonuclease from *Haemophilus aegyptius*. mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; tRNA, transfer RNA.

evidence is still lacking in *N. crassa.* The total coding capacity required for the products mentioned above only amounts to about one quarter of the *N. crassa* mitochondrial genome. As there is no indication for a large amount of spacer sequences in *N. crassa* mtDNA [6], this could mean that there are still many genes of which the function remains to be elucidated (for a review of the genetic function of organelle DNAs, see refs. 11 and 12). A physical map of the mtDNA would be useful to get a better insight in the organization of the structural RNA genes, to localize and confirm the existence of the genes for the already known mitochondrial translation products and to unravel the proposed regulatory function of mtDNA genes in the interaction with nuclear genes [13].

To construct a physical map of the mtDNA, we have made use of three restriction enzymes Bam HI, Eco RI and Hind III, which cleave the DNA in 5, 12 and more than 30 fragments, respectively. By analysis of partial fragments and digestions of isolated fragments with different endonucleases, we have constructed a complete overlapping map of all the Eco RI and Bam HI fragments, with a fragment-length range from 425 to 33 800 basepairs. Also we have determined the position of the largest Hind III fragment (14 500 basepairs) on this map as a starting point for finer analysis. While this work was in progress Bernard and Küntzel have published [14,15] a physical map of *N. crassa* mtDNA on which seven Eco RI fragments and the large Hind III fragment were ordered. The crucial part of their map was established by electron microscopy and denaturation mapping [3,14]. Our results confirm and complete the sequence. In addition we have extended the physical map with all the Bam HI fragments and obtained independent proof that the map must be circular. Some of our results have been summarized elsewhere [16].

Materials

Neurospora crassa strains. Wildtype strain E 5256, mating type A (ATCC no. 10815) and wildtype strain E 5297, mating type a (ATCC no. 10816), were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, where they are registered as C.B.S. 327.54 and C.B.S. 195.57, respectively.

Enzymes. Endonucleases Eco RI and Hind III were obtained from Miles Laboratories Inc. Endonuclease Bam HI and for some experiments also endonucleases Eco RI and Hind III, were obtained from New England Biolabs. Proteinase K was obtained from Merck.

Marker DNAs. Phage λ DNA was a gift from Mrs. F. Fase-Fowler (Laboratory of Biochemistry, section for Medical Enzymology, University of Amsterdam, The Netherlands). Hind II + III restriction fragments of *Saccharomyces carlsbergensis* mtDNA [17] were kindly donated by Dr. J.P.M. Sanders (Laboratory of Biochemistry, section for Medical Enzymology, University of Amsterdam, The Netherlands). Hae III restriction fragments of M13 DNA [18] were a gift from Dr. C.A. van den Hondel (Department of Molecular Biology, University of Nijmegen, The Netherlands).

Chemicals. Ethidium bromide was obtained from Serva, agarose from

SeaKem, *N,N'*-methylenebisacrylamide from Fluka, acrylamide from Merck and sodium *N*-lauroylsarkosinate from Koch-Light.

Methods

Isolation of mitochondria and extraction of mtDNA. Mitochondria were prepared as described previously [6]. The isolation of mtDNA from the mitochondria followed method A described in ref. 6, which includes: lysing DNAase treated mitochondria with *N*-lauroylsarkosinate, extraction with phenol and chloroform, RNAase A treatment, concentration and sucrose gradient centrifugation. From the sucrose gradient a fraction with a molecular weight higher than 10^7 was selected, dialyzed against 1 M NaCl, 10 mM Tris · HCl, 0.2 mM EDTA pH 7.4, and concentrated by pelleting in a SW41 rotor at 41.000 rev./min for 16 h at 0°C. The pellet was dissolved in 10 mM Tris · HCl, 0.2 mM EDTA, pH 7.4, at a concentration higher than 150 μg/ml.

φ29 DNA preparation. Phage φ29 and *Bacillus subtilus* 1G20 trp C2 were gifts from Dr. J. Buitenwerf (Department of Genetics, University of Groningen, Haren, The Netherlands). φ29 phage and φ29 phage DNA were isolated according to Streeck et al. [19] including a proteinase K treatment: 50 μg/ml in 0.5% *N*-lauroylsarkosinate, 10 mM Tris · HCl, 10 mM NaCl, 0.5 mM EDTA, pH 7.4, for 3 h at 37°C.

Restriction enzyme incubations. For analytical sixteen slot slabgels the standard incubation mixture usually contained 3 μg of mtDNA in a total volume of 30 μl with enough enzyme to obtain a complete digestion in 2 h at 37°C. For preparative gels with one slot an amount of 100 μg of mtDNA was usually employed. The incubation mixture for Eco RI and Hind III consisted of 10 mM Tris · HCl, 10 mM $MgCl_2$, 150 mM NaCl, 5 mM 2-mercaptoethanol, pH 7.6. For Bam HI: 6 mM Tris · HCl, 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol, 10 μg/ml relatin, pH 7.6. Double digestions with Bam HI and Eco Rl were carried out first with Bam HI alone in the Bam HI incubation buffer for 2 h at 37°C, whereafter NaCl $MgCl_2$ and Eco RI were added to the concentration required for the Eco RI incubation. The incubation was continued for 2 h at 37°C. Reactions were terminated by adding $\frac{1}{4}$ volume of a buffer containing 0.1 M EDTA, 70% sucrose, pH 7.4. Bromophenol blue was added (0.025%) to some mixtures as a mobility marker.

Slabgel electrophoresis. A gel system similar to the one described by De Wachter and Fiers [20] was used. The electrophoresis buffer contained 40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, pH 7.8. Composite slabgels (300 × 200 × 4 mm) were constructed as follows: the bottom of an empty gelchamber was placed in a small trough containing 10% acrylamide, 0.25% *N,N'*-methylene bisacrylamide in electrophoresis buffer to obtain a sealing layer of 10 mm. After polymerization, a layer of approx. 60 mm 3% acrylamide, 0.15% *N,N'*-methylene bisacrylamide in electrophoresis buffer was poured in the sealed chamber. After polymerization of this layer, 0.7% agarose, dissolved by autoclaving in electrophoresis buffer, was poured on top at 60°C. For analytical gels a perpex slotformer with sixteen 5 mm slots was used, for preparative gels one slotformer of 140 mm. Gels were run at room temperature at a constant voltage of 120 V for 15 h. After electrophoresis gels were stained

with 2 μg/ml ethidium bromide in electrophoresis buffer for 1 h.

Photography of slabgels. The gel was placed on a long wave ultraviolet lamp (U.V. Products Inc.) and photographed through a Kodak Wratten No. 23A filter with a Nikon F Camera and Kodak Panatomic X film. Suitable negatives were scanned with the Gilford 2400 spectrophotometer equipped with a linear transport mechanism.

Isolation of restriction fragments from slabgels. Appropriate bands were cut out from the agarose gel and the DNA was purified by the KI equilibrium centrifugation method of Blin et al. [21]. The DNA bands from the gradient tubes were extracted by sidewise puncture, extracted times with *iso*amylalcohol to remove the thidium bromide, diluted three-fold, made 0.1 M in sodium acetate (pH 5) and precipitated with 2 vol. of ethanol. After one night at −20° C, the precipitate was spun down in a SW41 rotor at 40 000 rev./min for 30 min at 0° C. The ethanol precipitate was dissolved in 10 mM Tris · HCl 0.2 mM EDTA, pH 7.4, and reprecipitated with ethanol. The second ethanol precipitate was dried in a vacuum dessiccator and dissolved in 10 mM Tris · HCl, 0.2 mM EDTA, pH 7.4, and used for incubations with restriction endonucleases. DNA from polyacrylamide gels was extracted and purified as described by Van den Hondel et al. [18].

Nomenclature of restriction fragments. Bands obtained from a complete digest with endonucleases Eco RI, Bam HI and Hind III are indicated with E, B and H, respectively, and bands from partial digests are indicated with Ep, Bp and Hp, respectively. EB and EH bands are the products of double digestions with endonucleases Eco RI plus Bam HI and Eco RI plus Hind III, respectively. Bands are numbered in order of increasing mobility. If one band contains two fragments, this is notated with a, b following the number of the band: e.g. E7a.

Results

Digestion of Neurospora crassa mtDNA with endonucleases Bam HI, Eco RI and Hind III

Construction of a restriction fragment map would preferably require an intact DNA as starting material. This, however, is not possible for *Neurospora crassa* mtDNA because preparative isolation gives only broken linear fragments [6,22], although 19 μm circular DNA can be observed with the electron microscope in osmotically shocked mitochondria [1]. For restriction enzyme analysis we have used fragments larger than 10^7 daltons taken from a sucrose gradient [6]. The DNA in this fraction contains all the sequences of total mtDNA, because the restriction fragment pattern is identical to the pattern obtained with total mtDNA, only the yield of high molecular weight restriction fragments is lower in total mtDNA. In Fig. 1 the results are shown of agarose gel electrophoresis of the digestion products obtained with endonucleases Bam HI (Fig. 1A), Eco RI (Fig. 1B) and Hind III (Fig. 1C). Scannings of the photographs of the gels are also presented in Fig. 1 to judge the molar proportion of the bands present. Prolonged incubation with the endonucleases and adding more enzyme to the incubation mixture did not alter the pattern of bands that can be observed in Fig. 1. From this we conclude that the bands in Fig. 1 represent the end products of digestion. The consequence of fragmented

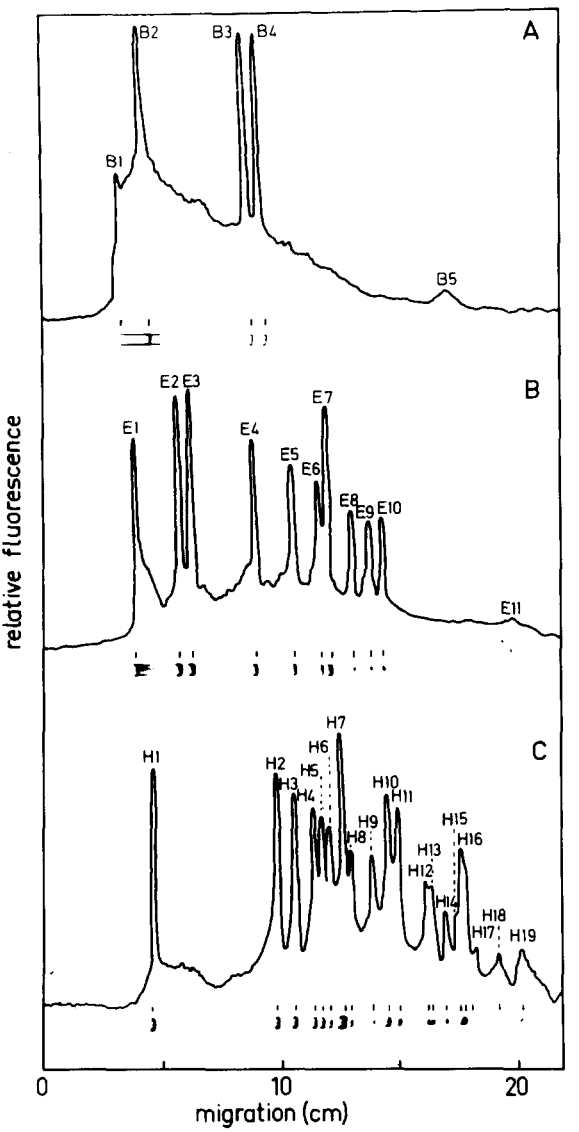

Fig. 1. Agarose gel electrophoresis of restriction fragments from *Neurospora crassa* mtDNA. For each digest a photograph of the gel is presented together with a tracing of this photograph. The bands are numbered in relation with increasing electrophoretic mobility (cf. Table I). 3 μg of mtDNA was incubated with each of the endonuclease and electrophoresed in analytical 0.7% agarose gel as described in Methods. A, complete digest with endonuclease Bam HI; B, complete digest with endonuclease Eco RI; C, complete digest with endonuclease Hind III.

DNA as starting material is clearly illustrated in Fig. 1A: complete digestion with endonuclease Bam HI gives five fragments. Because fragment B1 is longer than half a molecule (see Table I) it can only be observed as a faint sharp band, marking the upper size limit of a long tail of fragments that have only one or no cleavage site for Bam HI. Complete digestion with endonuclease Eco RI gives eleven bands (Fig. 1B). For the same reason as mentioned above, the molar amount of the large fragments E1 and E2 is lower than that of the

TABLE I

LENGTH OF THE RESTRICTION FRAGMENTS OF *NEUROSPORA CRASSA* mtDNA OBTAINED WITH ENDONUCLEASES Bam HI, Eco RI AND Hind III

Molecular weight values were obtained from the calibration curve in Fig. 2 and recalculated in basepairs on the basis of the sodium salt of DNA. Hind III fragments smaller than H19 are not included in this list (see text).

	$M_r \times 10^6$	Basepairs		$M_r \times 10^6$	Basepairs
Eco RI fragments			Hind III fragments		
E1	12.5	18 700	H1	9.7	14 500
E2	6.8	10.200	H2	2.6	3 900
E3	5.8	8 700	H3	2.3	3 500
E4	3.0	4 500	H4	2.0	3 050
E5	2.3	3 500	H5	1.9	2 900
E6	1.9	2 900	H6	1.8	2.750
E7a	1.8	2 750	H7a	1.65	2 470
E7b	1.8	2 750	H7b	1;65	2 470
E8	1.5	2 250	H8	1.58	2 370
E9	1.35	2 000	H9	1.35	2 020
E10	1.25	1 875	H10a	1.20	1 800
E11	0.3	425	H10b	1.20	1 800
			H11a	1.07	1 600
	40.3	60 550	H11b	1.07	1 600
			H12	0.87	1 300
Bam HI fragments			H13	0.83	1 250
			H14	0.73	1 100
B1	22.5	33 800	H15	0.66	980
B2	10.3	15 450	H16a	0.63	950
B3	3.1	4 650	H16b	0.63	950
B4	2.8	4 200	H17	0.56	840
B5	0.6	900	H18	0.43	640
			H19	0.28	420
	39.3	59 000		36.7	55 160

smaller fragments. Because the fluorescence intensity of band E7 is higher than can be expected on the basis of its fragment size (see tracing in Fig. 1B), we conclude that band E7 consists of two fragments with the same mobility in 0.7% agarose gels. This gives a total of twelve Eco RI terminal fragments. Complete digestion with endonuclease Hind III gives nineteen bands (Fig. 1C). There are, however, more than nineteen fragments, because some of the bands contain more than one fragment, as judged by their fluorescence intensity. From the scanning presented in Fig. 1C we conclude that band H7, H10, H11 and H16 contain at least two fragments each. In gels containing 10% acrylamide, eight additional smaller bands could be detected visually, but these have not been calibrated in length. In this way we arrive at a total of at least 31 Hind III fragments (Table I). This is about two and a half times as many as the fragments obtained with Eco RI, although the hexanucleotide sequences recognized by Eco RI and Hind III are expected to occur with equal frequency in a DNA with a random sequence of basepairs (Table II). We have looked for additional fragments in the 3% and 10% polyacrylamide layers of preparative gels (see Methods) on which approximately 100 μg of Eco RI or Bam HI

TABLE II

COMPARISON OF THE NUMBER OF RESTRICTION FRAGMENTS EXPECTED AND FOUND

Restriction endonuclease	Sequence recognized	Ref.	No. of fragments	
			Found	Expected *
Eco RI	G↓AATTC	23	12	19
Hind III	A↓AGCTT	24	>31 **	19
Bam HI	G↓GATCC	33	5	8

* Calculated from a random sequence of 60 550 basepairs with a G · C percentage of 40% [3,6].
** 31 fragments is a minimum estimate (see text).

digested DNA was loaded. As we have not observed smaller fragments than E11 and B5 in such gels, we have assumed that there are not more than 5 and 12 recognition sites on the mtDNA for Bam HI and Eco RI, respectively.

Calibration of fragment lengths

The restriction fragments larger than 10^6 daltons were calibrated in 0.7% agarose gels with intact phage λ DNA and its Eco RI restriction fragments [25] and intact phage ϕ29 DNA and its Eco RI restriction fragments [19] (Fig. 2A).

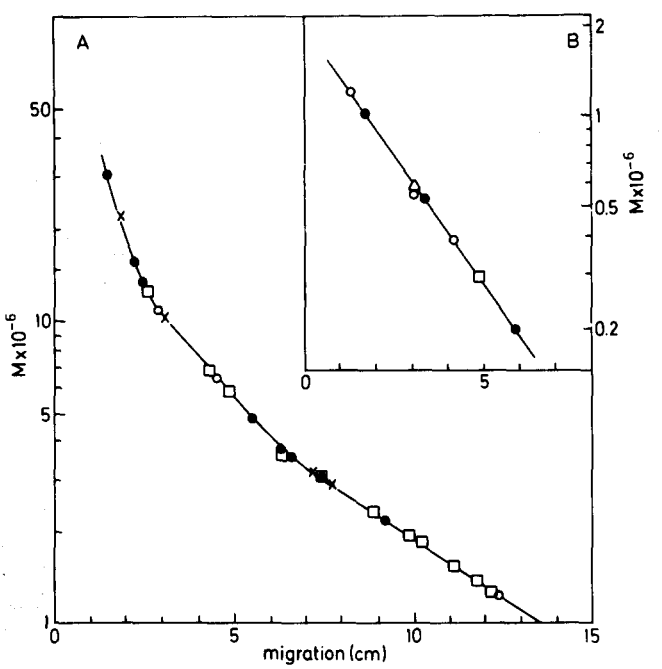

Fig. 2. Calibration of restriction fragment lengths. A. Calibration for 0.7% agarose gels. Mobility of the bands relative to the origin of the gel was measured in neighbouring lanes, containing standard length fragments and unknown fragments respectively. •, intact λ DNA and its Eco RI fragments, $X10^6$: 30.8, 15.8, 13.7, 4.7, 3.7, 3.5, 3.0 and 2.15 [25]; ○ intact ϕ29 DNA and its Eco RI fragments: 11.0, 6.5, 3.5 and 1.2 [19]; X, mtDNA Bam HI fragments; □, mtDNA Eco RI fragments. B. Calibration for 3% polyacrylamide gels. ○, ϕ29 Eco RI fragments. $X10^6$, 1.2, 0.56 and 0.4 [19]; •, M13 Hae III fragments: 1.08, 0.55 and 0.21 [18]; △, fragment B5; □, fragment E11. Hind III fragments of *Neurospora crassa* mtDNA have been calibrated against the derived lengths of Eco RI fragments from mtDNA and the lengths of ϕ29 Eco RI fragments.

As expected [26] large fragments run relatively fast in 0.7% agarose. This tendency persists in our gel system in a less pronounced way down to a fragment length of $3.7 \cdot 10^6$ daltons. Fragments smaller than $0.5 \cdot 10^6$ are sometimes difficult to detect in agarose gels. Therefore we have calibrated the smaller fragments in 3% acrylamide gels using the three smallest Eco RI fragments of ϕ29 DNA and Hae III fragments B, C and D of M13 DNA [18] as markers (Fig. 2B). The length of the restriction fragments calibrated in this way are presented in Table I. There are minor differences with the values presented earlier [16], where the lengths were calibrated with ϕ29 Eco RI fragments only. A further check on these lengths comes from redigestion of isolated terminal and partial fragments (see below), which gives consistent results within a margin of 5%.

The size of Neurospora crassa mtDNA

The sum of the lengths of the restriction fragments should be equal to the size of intact mtDNA, which corresponds to its kinetic complexity if there is no major gene repetition. The sum obtained with Eco RI fragments (Table I) gives an average of 60 550 ± 1300 (S.D.) basepairs, obtained with four different incubations on four different gels. This corresponds to a contour length of 19.5 ± 0.4 μm (27), which is in excellent agreement with the 19 μm previously measured in our laboratory with the electron microscope [1] and with the other length measurements of 20 μm [2,3]. The sum of 59 000 basepairs obtained with Bam HI fragments (Table I) is also in good agreement. The uncertainty in the calibration of the very large fragment B1, however, is about 10%, due to the poor resolution of bands in that region of the agarose gel. The sum of the Hind III fragments (Table I) is somewhat lower than the other two: 55 160 basepairs. This may be due to two reasons: firstly, we have not included fragments smaller than 400 basepairs in this sum, and in the second place, it is difficult to establish clearly the multiplicity of bands from a scanning of a photograph of a gel, especially in the lower molecular weight range (Fig. 1C), which may have led to an underestimation of the number of double bands.

Partial digestion with endonuclease Eco RI

Eighteen partial bands were reproducibly observed on digestion with a limiting amount of endonuclease Eco RI (Fig. 3). The length distribution of these partials is not favourable for ordering the fragments by calculation of the possible composition of the partial fragments. Even in the more favourable case of *Saccharomyces carlsbergensis* mtDNA [17] such a procedure may lead to errors [28]. We have therefore isolated on preparative scale some of the partial fragments and redigested these with Eco RI to observe their composition (Table III). Seven partials allowed straightforward conclusions: the observed fragments added up to their partial length within 5%. Three partial fragments gave variable results on separate redigestions with Eco RI. Partial Ep10 on redigestion gave E4, E7 and E6. As the sum of these three fragments is too large for Ep10 and is already assigned to Ep7b, we decided that Ep10 consisted of two different partial fragments with approximately the same mobility and that these two had the composition E4 + E7 and E4 + E6. Partial Ep7 on one occasion gave only E4, E6 and E7 on redigestion. On another redigestion Ep7

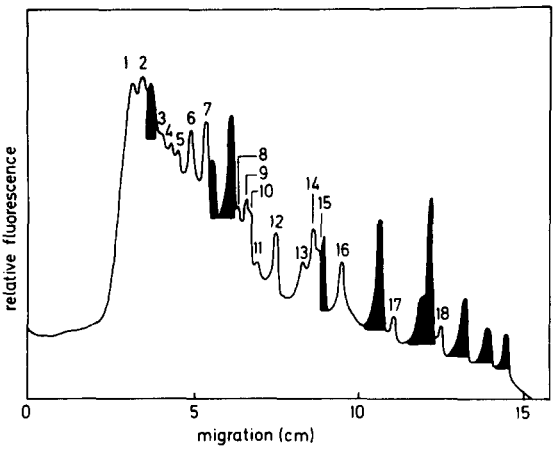

Fig. 3. Partial Eco RI digest of *Neurospora crassa* mtDNA. Electrophoresis was done in a 0.7% agarose gel. A scan of a photograph of this gel is presented. The Eco RI fragments are not numbered and are indicated with the black peaks. They correspond to fragments E1—E10 in Fig. 1B. Fragment E11 has run off the gel. Partial fragments are numbered in the same order as they appear in Table III, without the prefix Ep.

gave rise to E3 and E10 with strong fluorescence, E4, E6 and E7 with low fluorescence and E5 with very weak fluorescence. From this result we concluded that Ep7 is a double band and contains two partial fragments con-

TABLE III

LENGTH AND COMPOSITION OF THE PARTIAL FRAGMENTS OBTAINED WITH ENDONUCLEASE Eco RI (Fig. 3)

Eco RI fragment sequence				1—6—4—7—11—9—8—7—5—3—10—2
Partial fragment No.	Observed length (basepairs)	Calculated length (basepairs)	Difference (%)	Composition of partial fragments
Ep6a	12 500	12 200	—2.4	5—3
Ep6b *	12 500	12 075	—3.4	10—2
Ep7a *	10 700	10 575	—1.2	3—10
Ep7b	10 700	10 150	—5.1	6—4—7
Ep8 **	8 450	8 500	0.6	8—7—5
Ep9 **	7 800	7 825	0.3	4—7—11
Ep10a *	7 500	7 400	—1.3	6—4
Ep10b *	7 500	7 250	—3.3	4—7
Ep11 **	7 000	7 000	0	9—8—7
Ep12	6 000	6 250	4.2	7—5
Ep13 **	5 000	5 175	3.5	7—11—9
Ep14	4 800	5 000	4.2	8—7
Ep15 **	4 600	4 675	1.6	11—9—8
Ep16	4 100	4 250	3.7	9—8
Ep17	3 050	3 175	4.1	7—11
Ep18	2 550	2 425	—4.9	11—9

* The composition of these partials is based on isolation and redigestion with Eco RI and on calculation (see text).

** The composition of these partials is based only on calculation (see text). All other partials yielded after isolation and redigestion with Eco RI only the fragments presented in this table. The observed length of the partials was based on interpolation, using the Eco RI fragment lengths as internal calibration points.

sisting of E3 + E10 (Ep7a) and E4 + E6 + E7 (Ep7b), with a contamination of a third partial: representing E5 + E3, which is the composition of E6a. On close inspection of the gels, it was noted that partial Ep6 consists of two closely spaced bands, which cannot be isolated separately. On one redigestion it gave only E5 + E3 (assigned to Ep6a), but in another redigestion of Ep6 with Eco RI, seven fragments were produced: E2, E3, E5 and E10 with strong fluorescence and E4, E6 and E7 with weak fluorescence. If we ascribe E4 + E6 + E7 to contamination with Ep7b and assign E3 + E5 to Ep6a, then it follows that Ep6b may represent E2 + E10. The composition of five other partials has been derived solely on the basis of calculation (Table III), taking into account the order of fragments already established with the partial fragments described above. Partial fragments Ep8 and Ep9 give information on the polarity of the sequence E11-E9-E8 in between the two fragments E7. Additional indirect evidence for the polarity of E11-E9-E8 comes from the consideration that all the observed partials can be explained with the proposed sequence in Table III, whereas, if one reverses the polarity of E11-E9-E8, it is not possible to find a reasonably accurate composition for partial Ep9. Isolation of partial fragment Ep1 to Ep5 was not successful. It makes no sense to calculate probable compositions for these partials in view of the many, equally satisfactory, solutions within 5% accuracy.

Digestion of Hind III restriction fragment HI with endonuclease Eco RI

To get independent evidence for part of the Eco RI map (Table III), we have isolated the largest Hind III fragment H1 and digested it with endonuclease Eco RI (Fig. 4). The large fragment EH1 (Table IV) can be derived from E1, E2 or E3. From digestion of these Eco RI fragments with Hind III it followed, that only E1 contains a fragment of corresponding length (data not show). The two partial bands observed in Fig. 4, which disappear on prolonged incubation with Eco RI, permit us to calculate the sequence of the four Eco RI fragments contained in HI (Table IV). In terms of the Eco RI map this corresponds to the sequence E1-E6-E4, confirming part of the Eco RI map,

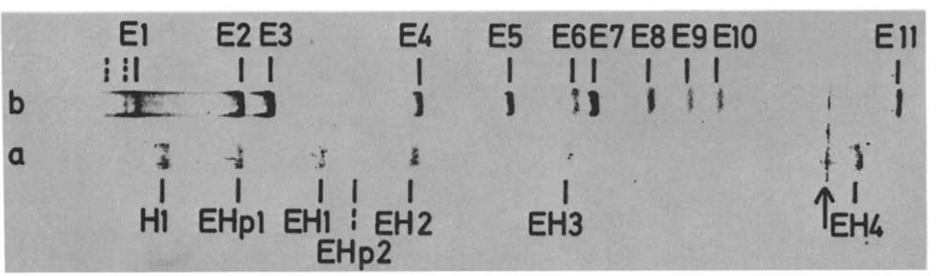

Fig. 4. Partial Eco RI digest of Hind III fragment HI. Hind III fragment HI was isolated from a preparative 0.7% agarose slabgel as described in Methods and after partial digestion with Eco RI, run on an analytical slabgel together with an Eco RI digest of mtDNA. The arrow marks the border between the 0.7% agarose part of the gel (left) and the 3% polyacrylamide part of the gel (right). Lane a, partial Eco RI digest of HI. The nomenclature of the bands is the same as in Table IV and is explained in Methods. Partial band EHp2 is too faint for reproduction and its position is indicated only. Lane b, Eco RI digest of mtDNA. The two bands, which migrate slower than E1, are the partial fragments Epl and Ep2 (cf. Fig. 3).

TABLE IV

DIGESTION OF HIND III FRAGMENT H1 WITH ENDONUCLEASE Eco RI (Fig. 4)

Fragment sequence				EH1—EH3—EH2—EH4
Fragment No.	Observed length final fragments (basepairs)	Observed length partial fragments (basepairs)	Calculated length partial fragments (basepairs)	Composition of partial fragments
EHp1	—	9 750	9 350	EH1—EH3
EH1	6 450	—	—	EH2—EH4
EHp2	—	5 250	5 300	
EH2 = E4	4 500	—	—	
EH3 = E6	2 900	—	—	
EH4	[800	—	—	
Total length	14 650			
Expected length	14 500			

based on partial analysis and providing the overlap of H1 with the Eco RI map.

Partial digestion with Bam HI

Digestion of the mtDNA with limited amounts of endonuclease Bam HI gives a simple pattern: three partial fragments can be observed in Fig. 5. The composition of Bp2 and Bp3 can be accurately determined by calculation (Table V) in view of the very limited possibilities, but the composition of Bp1 is ambiguous: it may be the sum of B2 + B3 + B5 or of B2 + B4 + B5. In the first case we arrive at map A in Table V, in the second case at map B in Table V. The latter would require a partial fragment with a length of 8850 basepairs (B3 + B4), which we have never observed. However this is not conclusive and

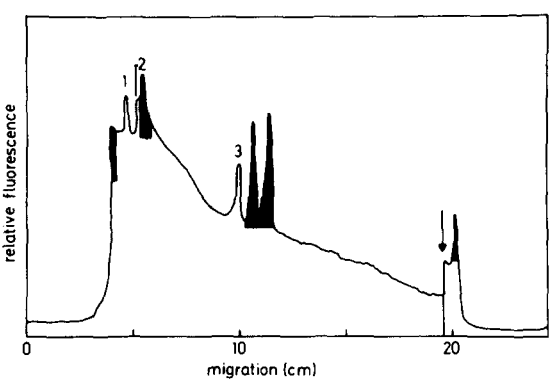

Fig. 5. Partial Bam HI digest of *Neurospora crassa* mtDNA. Electrophoresis was done in a composite slabgel: the arrow marks the border between the 0.7% agarose part (left) and the 3% polyacrylamide part (right) of the gel. A scan of a photograph of this gel is presented. The baseline of the 3% polyacrylamide gel scan is actually at a lower level due to the greater transparency of such gels as compared to agarose gels. The Bam HI fragments are not numbered and are indicated with the black peaks. They correspond to fragments B1—B5 in Fig. 1A. Partial fragments are numbered in the same order as they appear in Table V, without the prefix Bp.

TABLE V

LENGTH AND COMPOSITION OF THE PARTIAL FRAGMENTS OBTAINED WITH ENDONUCLEASE Bam HI (Fig. 5)

Possible sequences deduced from partial analysis *			(A) 1—4—5—2—3 (B) 3—4—5—2—1
Partial fragment No.	Observed length (basepairs)	Calculated length (basepairs)	Composition of partial fragments
Bp1	21 800	(A) 21 000	5—2—3
		(B) 20 550	4—5—2
Bp2	16 500	16 350	5—2
Bp3	5 050	5 100	4—5

* See text.

to decide which of the two sequences is the correct one, additional data were collected (see below).

Digestion of the Eco RI fragments with endonuclease Bam HI

To decide which of the two possible Bam HI maps in Table V is correct and to align that map with the Eco RI map in Table III, we have isolated all the Eco RI fragments, except E11 and digested these with endonuclease Bam HI. From these redigestions it could be concluded that E2, E3, E4, E8, E9 and E10 contain no cleavage site for Bam HI (Table VI). The five Bam HI recognition sites are located on the Eco RI fragments E1, E5, E6 and E7 (Fig. 6 and Table VI) in the following way: E1 is cleaved once, giving rise to fragment EB1 and EB4 (Fig. 6, lane d) of which EB4 has the same mobility as E4. E5 yield EB5 (Fig. 6, lane i), which is only 200 basepairs shorter than E5 itself (Table VI) implying the existence of a small fragments EN14, which is too short to be observed in our gel system. The digestion pattern of E6 and E7 is rather complicated: E6 is shortened by 150 basepairs resulting in EB6, with the same mobility as E7 (Fig. 6, lane g) and EB15 which is too short to be observed. One of the two fragments in band E7 is not cleaved by Bam HI, but the other contains two cleavage sites, giving rise to the three small fragments EB10, EB11 and EB12 (Fig. 6, lane e and Table VI), of which EB11 has the same mobility as B5. The two corresponding partial fragments EB10 + EB11 and EB11 + EB12 have been detected in another gel (data not whown). It could be argued that due to contamination of E7 with E6, the three small fragments EB10m EB11 and EB12 originate from E6 and that both E7a and E7b are not cleaved by Bam HI. Electrophoresis of E6 and E7 on the same gel (Fig. 6, lanes h and f) however, shows that the cross contamination is negligible. By comparing the double digestion products obtained with Eco RI plus Bam HI with an Eco RI digest alone)Fig. 6, lanes a and c), we conclude that E11 is not cleaved by Bam HI. As expected from the digestion of Eco RI fragments with Bam HI, bands EB4 and EB6 are double and contain two fragments each (Fig. 6, lane a). This is confirmed by and optical scan of lane a in Fig. 6 (data not shown).

TABLE VI

LENGTH OF THE FRAGMENTS OBTAINED AFTER INCUBATION OF ISOLATED Eco RI FRAGMENTS WITH ENDONUCLEASE Bam HI (Fig. 6)

Bam HI X Eco RI fragment No. (Fig. 6)	Eco RI fragments digested with Bam HI										
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11
EB1	14 500										
EB2		10 200									
EB3			8 700								
EB4	4 500			4 500							
EB5					3 300						
EB6						2 750	2 750				
EB7								2 250			
EB8									2 000		
EB9										1 875	
EB10							975				
EB11 = B5							900				
EB12							765				
EB13											425
EB14					200 *						
EB15						150 *					
Total length (basepairs)	19 000	10 200	8 700	4 500	3 500	2 900	5 390	2 250	2 000	1 875	425
Expected length (basepairs)	18 700	10 200	8 700	4 500	3 500	2 900	5 500 **	2 250	2 000	1 875	425

* Not observed, but calculated (see text).

** The sum of E7a and E7b.

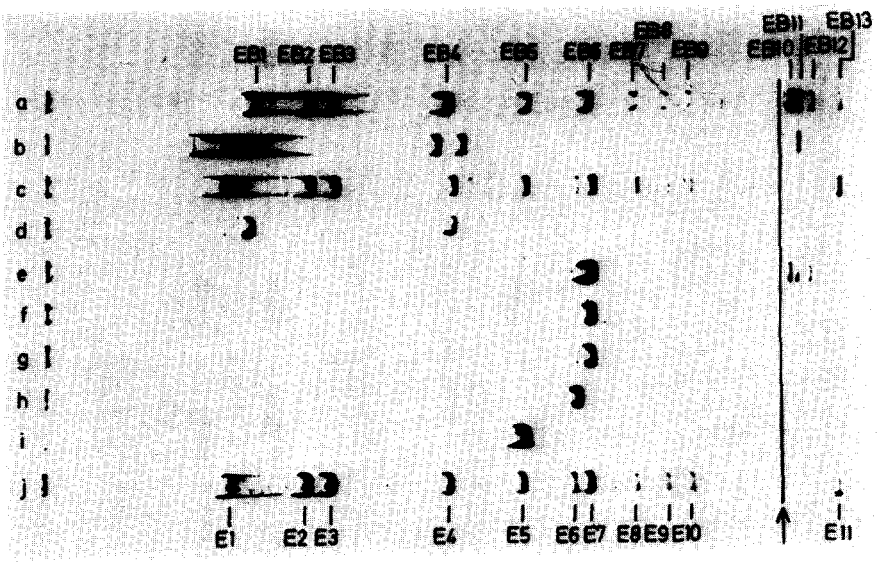

Fig. 6. Digestion of Eco RI fragments with endonuclease Bam HI. Electrophoresis was done in a composite slabgel: the arrow marks the border between the 0.7% agarose part (left) and the 3% polyacrylamide part (right) of the gel. The polyacrylamide part has been exposed for a longer time to increase the visibility of the small fragments. Lane a, a complete double digestion of mtDNA with Eco RI and Bam HI (see Methods). The bands are numbered in the same order as they appear in Table VI. Lane b, a complete digest of mtDNA with Bam HI. Lanes c and j, a complete digest of mtDNA with Eco RI. The bands are numbered below lane j in the same order as in Fig. 1B. Lane d, fragment E1 digested with Bam HI. Lane e, fragment 57a + E7b digested with Bam HI. Lane f, fragment E7a + E7b without redigestion. Lane g, fragment E6 digested with Bam HI. Lane h, fragment E6 without redigestion. Lane i, fragment E5 digested with Bam HI.

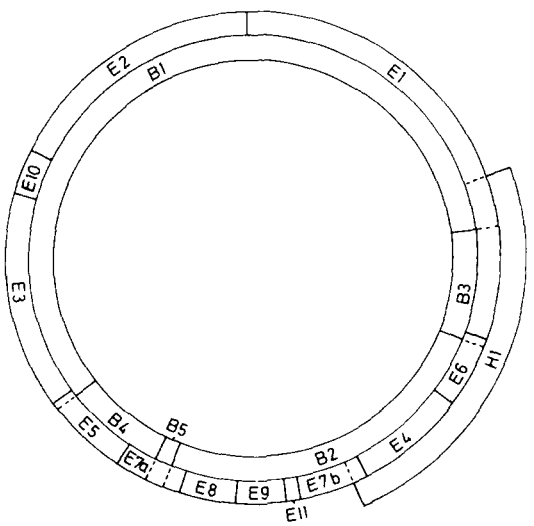

Fig. 7. Restriction fragment map of *Neurospora crassa* mtDNA. For the construction of this map, the Eco RI fragment sequence of Table III was combined with the Bam HI fragment sequence A in Table V and the Eco RI fragment sequence of fragment HI in Table IV. Outer segment, Hind III fragment HI. Inner ring, Bam HI fragments. Middle ring. Eco RI fragments are separated by solid lines. The Bam HI and Hind III cleavage are indicated with broken lines.

TABLE VII

DIGESTION OF HIND III FRAGMENT H1 WITH ENDONUCLEASE Bam HI

Fragment sequence:				HB1—HB2—HB3
Fragment No.	Observed length final fragments (basepairs)	Observed length partial fragments (basepairs)	Calculated length partial fragments (basepairs)	Composition of partial fragments
HBp1	—	12 500	12 650	HB1—HB2
HB1	8 000	—	—	
HBp2		6 400	6 400	HB2—HB3
HB2 = B3	4 650	—	—	
HB3	1 750			
Total length	14 400			
Expected length	14 500			

Construction of the complete cleavage map

Once the place of the Bam HI recognition sites on the Eco RI fragments is known, it is immediately clear that a correct overlap of the Eco RI map in Table III with the two Bam HI maps in Table V is only possible with map A in Table V. To get a full circular overlap, we have made the sum of the Bam HI fragment lengths equal to the sum of the Eco RI fragments lengths (Table I) by adding 1550 basepairs to fragment B1. This falls within the 10% error of the length calibration of B1. A complete restriction fragment map of *Neurospora crassa* mtDNA is presented in Fig. 7. As predicted from this map, digestion of the isolated Hind III fragment H1 with Bam HI yields three fragments: HB1, HB2 and HB3 of which HB2 is identical to B3 (Table VII).

Up till now we have assumed that the restriction fragment map would be circular in view of the electron microscopic observation of circular mtDNA molecules [1—3]. Without this assumption, however, we can now conclude from the overlap of the Eco RI map and the Bam HI map that the complete map must be circular: there are two Eco RI sites, one on B3 and one on B1, which are so close to Bam HI sites that they yield on double digestion two small unobserved fragments (EB14 and EB15 in Table VI). It is not possible to consider these two Eco RI sites as termini of a linear map, because these sites have been located within Eco RI partial fragments. (Ep6a in Table III and EHp1 in Table IV).

Discussion

The most important reason for constructing a physical map of a DNA is to provide a basis for studying the arrangement of genes on the genome. The map presented in Fig. 7 has already proven its usefulness for the localization of the rRNA genes (for a preliminary account see ref. 7). Moreover independent evidence on the circularity of the mtDNA is obtained. From the fact that all fragments add up to the molecular weight expected from the length measurements of the DNA [1—3], it can be concluded that major sequence repetition and intermolecular heterogeneity is absent. Therefore, the sequence complexity

of *Neurospora crassa* mtDNA corresponds to $4 \cdot 10^7$ daltons. Direct measurements of the kinetic complexity of *N. crassa* mtDNA resulted in values about 50% higher [6,29]. This is probably an overestimation because the values obtained by renaturation measurements vary depending on the conditions used [30].

Concerning the construction of the map there remain a few points which need further discussion:

(1) The polarity of the sequence E8-E9-E11 has only been established indirectly. Analysis of the Bam HI digestion products of Eco RI partial fragments Ep17 and Ep14 (Table III) will provide more direct evidence.

(2) The sequence E3-E10-E2 was inferred by combining data from redigestion of partial fragments and from calculation. More direct evidence would come from partial digestion of fragment B1 with Eco RI.

(3) We have observed that one of the two E7 fragments has nearly the same mobility as fragment E6 in 3% acrylamide gels. This could be caused by a relative high A · T content of this fragment, because it has been reported that DNA fragments with a high A · T content migrate slower in acrylamide gels than fragments of the same size and rich in G · C [31]. Isolation of the separated E7 fragments and digestion with Bam HI, revealed that the putative A · T-rich fragment contains the two G · C-rich Bam HI cleavage sites (cf. Table II). This identifies in an unequivocal way fragment E7a in the map presented in Fig. 7.

(4) Calibration of the restriction fragments of *N. crassa* mtDNA with phage λ and φ29 DNA (Fig. 2) gave internally consistent results. However, when we performed a calibration against Hind II + III restriction fragments of *Saccharomyces carlsbergensis* mtDNA [17], we obtained a set of length values significantly lower for all fragments smaller than E3, thus preventing the construction of any overlap between the Eco RI map and the Bam HI maps. This may be explained when we assume that DNA fragments with such a high A · T content as yeast mtDNA (82% [32]) migrate slower than expected even in 0.7% agarose gels.

The restriction fragment map in Fig. 7 partly confirms and extends the incomplete physical map of *N. crassa* mtDNA presented by Bernard et al. [15], which is based partly on denaturation mapping [3,14] and partly on restriction enzyme analysis [14,15]. The differences that arise are the following:

(a) Bernard et al. [15] place fragment E9 in between E2 and E3. This may be due to the fact that in their case the composition of the smaller Eco RI partials has been inferred only from calculation, which did not clearly resolve the sequence between E7a and E7b.

(b) Our Eco RI fragment pattern of *N. crassa* wild type strain 5256 mtDNA is identical to the Eco RI fragment pattern of wild type strain 7A mtDNA used by Bernard et al. [15]. Our derived calibration of the length of the Eco RI fragments gives a sum in agreement with the electron microscopic measurements from our laboratory [1]. This sum is $3.5 \cdot 10^6$ daltons lower than the electron microscopic measurement of Bernard et al. on Eco RI fragments [15,22]. There is, however, no significant difference in the relative length of the fragments.

(c) The mtDNA of the identically labelled wildtype strain 5256, used by Bernard et al. [15] gives a slightly different Eco RI restriction fragment

pattern: fragment E5 is significantly shorter and migrates between E7 and E8 [15]. It would be interesting to check whether this is caused by an internal deletion in E5, in which case B4 would also be shorter (Fig. 7) or by two point mutations, in which case the Eco RI recognition site separating E3 and E5 could have a location on B4. It will, however, be difficult to resolve whether these two strains 5256 are really of the same origin, in which case the difference in the mtDNA could be explained by a recent mutation, or whether the two strains are of different wild-type origin and are mislabelled. Small differences are not uncommon in the patterns of large restriction fragments obtained from mtDNAs of different wild-type origin and have been noted for example in yeast mtDNA [28].

We have compared the Eco RI restriction fragment pattern of *N. crassa* wild type strain 5297 with that of strain 5256. No differences in terminal and partial fragment lengths were found. But in the mitochondria of strain 5297 a population of small circular DNA molecules, heterogeneous in length, has been detected by electron microscopy together with a few full-length 19 μm [1]. The restriction fragment pattern then would suggest that these small circles contain random sequences of a circular 19 μm DNA. In one preparative CsCl-ethidium bromide gradient of 5297 mtDNA we have isolated a small amount of closed circular DNA with a mitochondrial density. It is very likely that this fraction contained small circles, because we found it impossible to isolate 19 μm circular DNA in the same way from strain 5256 (unpublished results). Repeated attempts to isolate more of this circular mtDNA from strain 5297 to examine directly its composition have, however, failed. Possibly the occurrence of the heterogeneous small circles in strain 5297 mitochondria is a transient phenomenon, influenced by, as yet, unknown factors. Without further information on this point, we conclude that the Eco RI restriction fragments of mtDNA from strain 5297 are derived from full-length 19 μm circles.

Acknowledgements

We are indebted to Dr. J.G.G. Schoenmakers (Department of Molecular Biology, University of Nijmegen, The Netherlands) who allowed us to perform some initial experiments in his laboratory, and to B. Tebbes for the photography of the gels. The technical assistance of Hennie Bakker during the final stage of the experimental work is also gratefully acknowledged. This work was supported in part by a grant from the Netherlands Foundation of Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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