SPECIFIC FRAGMENTATION OF MITOCHONDRIAL DNA FROM NEUROSPORA CRASSA BY RESTRICTION ENDONUCLEASE ECO R I

U. Bernard, E. Bade and H. Küntzel

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, and Institut für Mikrobiologie, Abteilung Molekulare Genetik, GSF, 34 Göttingen, Germany.

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SUMMARY: Linear, size-heterogenous mitochondrial DNA from Neurospora crassa was cleaved by the restriction endonuclease Eco R I into eleven specific fragments. According to their contour lengths the fragments have molecular weights between 1.1 and 14 x 10^6. The sum of the fragments lengths is identical with the contour length (19.8 µm, 41 x 10^6 daltons) of the few circular molecules detectable in purified DNA preparations.
The results suggest sequence homogeneity of mitochondrial DNA and further demonstrate that restriction enzymes can be used to establish a physical map of an unspecifically-fragmented DNA molecule.

Introduction

Restriction endonuclease Eco R I recognizes the relatively large sequence ...G↓AATTC... / ...CTTAA↑G... and therefore introduces only a limited number of double strand breaks into most DNA molecules. Together with the restriction endonuclease Hind III this enzyme represents one of the most useful tools for physical and transcriptional mapping of DNA and for studying homology between related DNA molecules (1).

The technique of specific fragmentation has not only been applied to viral DNA (1) but also to animal mtDNA[+] (molecular weight 10 x 10^6) in order to localize the origin and direction of replication (2,3) and to follow the inheritance of mtDNA in donkey/horse crosses (4).

In contrast to animal mtDNA which can be easily isolated in intact circular form, attempts to physically map the four- to five-times larger mitochondrial genome of lower eukaryotes like yeast and Neurospora have been unsuccess-

[+]Abbreviation: mtDNA, mitochondrial DNA.

ful so far, mainly because no one has been able to isolate this DNA in intact form and to separate single strands (5). Purified mtDNA from Neurospora consists of linear molecules of rather even size distribution between 0.1 and 20 µm contour length, and only about 1 % of the DNA is recovered in circular form (19.8 µm, mass = 41 x 10^6 daltons). We demonstrated by denaturation mapping that the linear molecules derive from the circular genome by random breaks during isolation (6).

Here we report that such a randomly-fragmented DNA can be successfully analyzed by the restriction endonuclease Eco R I.

Methods

Growth of Neurospora crassa (wild type strain 5256) and purification of

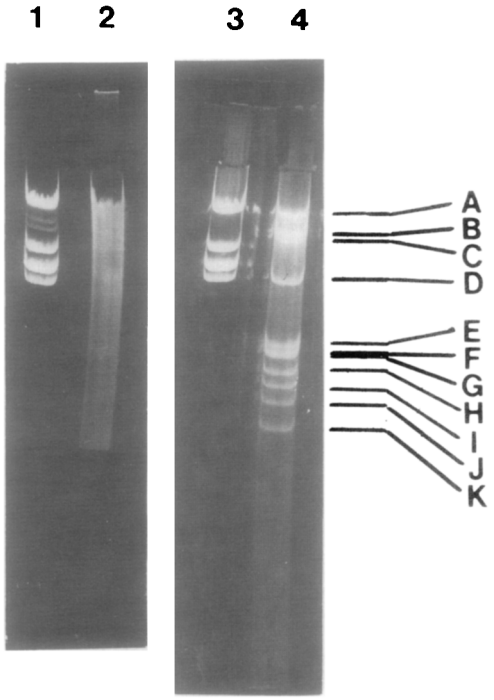

Fig. 1: Agarose gel electrophoresis of Neurospora mtDNA before (gel 2) and after (gel 4) treatment with Eco R I. The resolution of fragments F and G is sometimes difficult to visualize. Eco R I fragments of lambda DNA were used as standards (gels 1 and 3). Gel 1 contains two bands of partially digested material between the upper two main bands.

mitochondria has been described (7). mtDNA was purified by phenol extraction and CsCl density gradient centrifugation according to Nass (8). Peak fractions of the CsCl gradient were dialyzed against 1 x standard saline citrate (150 mM NaCl, 15 mM sodium citrate),10 mM Tris-HCl pH 8, 1 mM EDTA, and centrifuged in the above buffer (1:10 diluted) at a concentration of 27 A_{260}/ml.

Restriction endonuclease Eco R I was purified by a slightly modified procedure described by Yoshimori (9). The preparation contained neither detectable exonuclease activity nor other endonucleases because prolonged digestion of lambda DNA did not alter the pattern of specific fragments in gels. mtDNA (2.7 µg/ml) was incubated for 12 hours at 37° in a medium containing 0.1 M Tris-HCl pH 7.6, 0.01 M $MgSO_4$, 0.02 x standard saline citrate and 7 mM β-mercaptoethanol with 5-fold the amount of enzyme needed to completely digest lambda DNA as determined by the disappearance of bands of partially digested material.

Analytical agarose gel electrophoresis in the presence of ethidium bromide was performed essentially as described (10). DNA was recovered from gel bands by

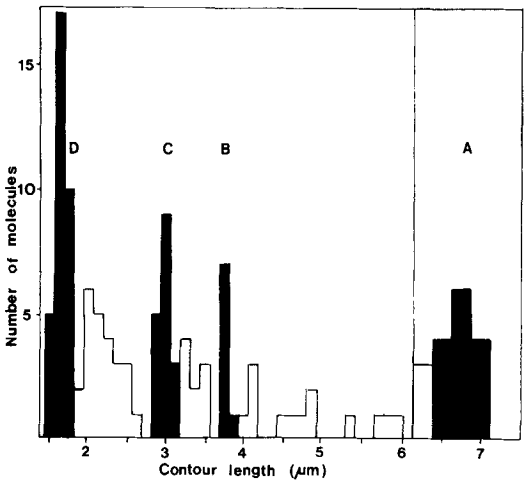

Fig. 2: Contour length histogram of mtDNA digested with Eco R I. Only fragments longer than 1.5 µm are shown. The bar width to the left of the vertical line is 0.14 µm, to the right 0.28 µm. The contour lengths of the black areas were used for calculating the data of Table 1 (fragments A to D).

electrophoresis, precipitated with ethanol and dissolved in 0.2 M ammonium acetate. Electron microscopy procedures were performed as described (11).

Results and Discussion

Fig. 1 shows the results of agarose gel electrophoresis of Neurospora mtDNA before and after cleavage with Eco R I. The absence of defined bands in the untreated sample (gel 2) confirms our previous finding that circular mtDNA is almost completely and randomly degraded during isolation (6). However one expects not only the few intact molecules but also the larger unspecific fragments to be processed by the restriction enzyme into specific fragments. Indeed, the endonuclease-treated sample (gel 4) exhibits eleven sharp bands designated by letters A to K in the order of decreasing size (12). The DNA sample treated with Eco R I was also analyzed by electron microscopy. Fig. 2 shows a histogram of the contour length range between 1.5 and 7 µm. The contour lengths of the four maxima A to D, which are not found in the untreated sample, correspond to molecular weights of 14, 7.7, 6.1 and 3.4 x 10^6 (13). From the calibration plot of Fig. 3, it is obvious that the four maxima of the histogram represent the four largest fragments A to D (Fig. 1, gel 2),

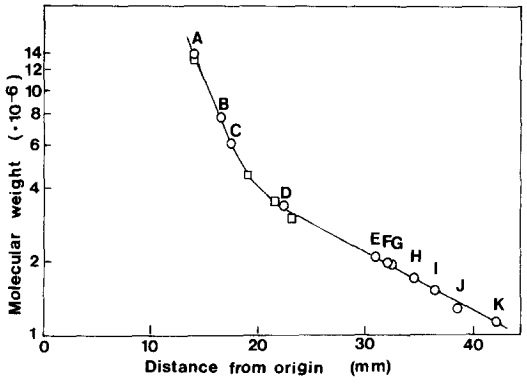

Fig. 3: Molecular weights of Eco R I fragments of Neurospora mtDNA (O) and lambda (□) plotted against the electrophorectic mobility in agarose gels.

because the same molecular weights are obtained from the electrophoretic mobility relative to lambda DNA fragments (14). The maxima corresponding to bands E to K were not sufficiently resolved in the histogram; the contour lengths of these fragments were therefore measured after re-isolation from gel bands. The molecular weights of all eleven fragments are summarized in Table 1. Since an unspecifically-fragmented DNA was used for sequence-specific endonuclease cleavage, it is clear that not all DNA molecules found in the histogram maxima or

Table 1: Mean molecular weights of circular mtDNA and of Eco R I fragments of linear mtDNA.

Species	number of measured molecules	molecular weight (x 10^6)
circular DNA	22	41.0 ± 1.9
fragments:		
A	14	14.0 ± 0.4
B	8	7.7 ± 0.1
C	17	6.1 ± 0.2
D	32	3.4 ± 0.2
E	13	2.1 ± 0.1
F + G	16	2.0 ± 0.1 (2 x)
H	12	1.7 ± 0.1
I	14	1.5 ± 0.1
J	16	1.3 ± 0.1
K	21	1.1 ± 0.1
		42.9 ± 0.6

The molecular weights were calculated from the contour lengths using the factor 1 μm = 2.07 x 10^6 (13). The contour lengths of fragments A to D were taken from the histogram maxima of Fig. 2, fragments E to K were measured after re-isolation from gel bands (Fig. 1).

recovered from gel bands are specific fragments. However, denaturation mapping of the four largest fragments revealed that more than 80 % of the molecules within a given size class had identical sequences (Bernard, Pühler and Küntzel, manuscript in preparation).

In Fig. 3 we have plotted the electrophoretic mobility of all eleven mtDNA fragments and four lambda DNA fragments (14) against the log of their molecular weights. This plot is linear for fragments smaller than 1.6×10^6 daltons: resolution then decreases with increasing fragment size.

The results reported here demonstrate sequence homogeneity of mtDNA because the sum of the molecular weights of the eleven restriction fragments is not significantly larger than the molecular weight of the circular molecule (Table 1). The results further demonstrate that the two techniques of specific fragmentation and denaturation mapping can be applied to establish a physical map of an unspecifically-degraded DNA molecule. The positions of the four largest fragments A to D on the circular genome have already been determined by denaturation mapping (Bernard et al., manuscript in preparation). By molecular hybridization of restriction fragments with RNA it should be possible to map mitochondrial genes coding for rRNA, tRNA and mRNA.

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