Mitochondrial DNA from *Podospora anserina*

III. Cloning, Physical Mapping, and Localization of the Ribosomal RNA Genes

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Summary. *EcoRI* fragments of the 94 kilobase mitochondrial DNA (mtDNA) from young, wild type *Podospora anserina* were cloned into the *EcoRI* site of the *E. coli* plasmid vector pBR325. A complete *EcoRI* clone bank was developed, containing all 16 of the *EcoRI* fragments from the native mtDNA. Restriction endonuclease maps for the enzymes *SalI*, *XhoI*, *BamHI*, *EcoRI*, *BglII*, and *HaeIII* were constructed from the analysis of single, double, and triple restriction digests of cloned and native mtDNA. In constructing the maps data were refined by extensive Southern analysis of the native genome hybridized to cloned DNA probes. Restriction maps were analyzed and permitted us to locate the origin of mtDNA derived from senescent cultures.

Both the large and small rRNA genes were then localized on these restriction maps using Southern and Northern blot analysis. We have shown the large rRNA locus to lie within a 10.8 kb region of *EcoRI* fragments E5 and F7, and the small rRNA locus to lie on a 5 kb subfragment of *EcoRI* fragment E1. The limit of separation between these two loci was determined to be between 6 and 9 kb.

Surprisingly, when electrophoresed in agarose-CH$_3$HgOH gels, the large rRNA was found to be 3.8 kb long, 500 bases longer than that from the very closely related *Neurospora crassa*, making it the largest rRNA yet described.

Introduction

The ascomycete fungus *Podospora anserina* is characterized by a program of cellular death. From the point of spore germination, mycelia grow continuously for a period and then the entire hyphal population begins to die. Exactly when this cellular lethality occurs depends upon mutations carried by mycelia, upon the race of *Podospora* in question, and for some races, upon the mating type. Some races cease to grow after only 15-20 cm of growth, and in chloramphenicol resistant mutants the life span is greatly increased (Belcour and Begel 1980). The genetic control of cellular lethality (senescence) has been under investigation for many years (Rizet 1953; cf. review by Esser 1974), and it was established genetically that the point when senescence occurred was under maternal or cytoplasmic control (Rizet 1957; Smith and Rubenstein 1973). That mitochondria were responsible for the control of the timing of senescence was established genetically (Belcour and Begel 1977, 1978, 1980), and additional support for the involvement of mitochondria was derived by studies on the inhibition of mitochondrial function and the effect on the timing of senescence (Tudzynski and Esser 1977; Belcour and Begel 1980). For example, the inhibitors of mitochondrial function, chloramphenicol and ethidium bromide, were shown to delay the timing of senescence (Belcour and Begel 1980).

On the basis of genetic evidence a model for the role of mitochondria in the development of senescence was proposed (Belcour and Begel 1978). By analogy to suppressiveness in the yeast *Saccharomyces* this model proposed the case in which mtDNA was subject to an excision-amplification mechanism leading to mitochondrial deficiency and to a rho minus-like state of the mtDNA. Other models of course have been proposed (Esser et al. 1980). Physical evidence for the excision-amplification hypothesis sprang from the demonstration of the presence of multimeric, circular DNA in the mitochondria from senescent mycelia (Cummings et al. 1979b). This DNA possessed a unit repeat size which was detected by *HaeIII* restriction endonuclease analysis (Cummings et al. 1979b) and was later referred to as Sen-DNA (Jamet-Vierny et al. 1980). Sen-DNA was subsequently shown to be derived from the mitochondrial genome (Jamet-Vierny et al. 1980; Cummings et al. 1980; Belcour et al. 1981; Kuck et al. 1981) by the use of Southern blot analysis. Senescent specific mtDNA clones were then constructed (Cummings et al. 1980) in order to determine the origin of Sen-DNA on the non-senescent genome, and a number of Sen-DNAs have been located on a *HaeIII* digest of the non-senescent mitochondrial genome.

As a prelude to examining the molecular details of the involvement of mitochondria in the development of senescence it is necessary to devise physical and genetic maps of the young, non-senescent genome. In this paper, we present the construction of a mtDNA clone bank, restriction maps for the enzymes *SalI*, *XhoI*, *EcoRI*, *BamHI*, *BglII*, and *HaeIII*, and the localization of the ribosomal RNA genes. We discuss the restriction maps in the context of the origin of Sen-DNA, and we make the surprising observation that the large rRNA from *Podospora* mitochondria is 3,800 bases long.

Material and Methods

Culture Conditions

*Podospora anserina* race s of the + mating type was used throughout. Mycelia were propagated at 27°C on solid agar plates supplemented with corn meal extract (Belcour and Begel 1977). Bulk growth was in liquid corn meal extract containing 5 g/l of Difco yeast extract, and cultivation was conducted as de-
scribed by Cummings et al. (1979a). Because we wish to establish a young, non-senescent reference point, all liquid cultures were initiated from mycelia that had grown no more than 5 cm from the point of spore germination.

Preparation of Mitochondria and Mitochondrial DNA
Mitochondria were prepared essentially as described by Cummings et al. (1979a). Important modifications were as follows: After filtration and washing, 200–250 g wet weight of mycelia were treated with 2 x 10^6 units of β-glucuronidase (Sigma) at 27°C with gentle rotation for 60 min followed by 30 min of treatment without rotation. Thirty to forty grams of pelleted mycelia and protoplasts were disrupted in 100 ml of isolation buffer (50 mM KH2PO4, pH 7.6, 0.5 mM EDTA, 0.66 M sucrose) at 4°C. This allowed the interface between steps to diffuse slightly or "blur". Mitochondria were banded at the interface zone and the bulk of contaminating nuclei sedimented through this interface. The mitochondrial band was harvested from the top, diluted in 10 mM Tris, pH 7.6, 100 mM NH4Cl poured 34 h before use and stored at 4°C. This preparation of mitochondria on DAPI-CsCl gradients exactly as described before. DNA was prepared from mitochondria by homogenization in a Tekmar Tissuemizer (Segal Scientific, Inc.) at a setting of 37 for 1 min at 0–4°C. The homogenate was diluted with 100 ml of isolation buffer and shaken by hand (gently) for 1 min. Gridding with the Tekmar Tissuemizer in the full 200 ml of isolation buffer produced less efficient isolation of mitochondria. The homogenate was then filtered through nylon gauze to remove mycelial debris, and mitochondria were isolated from the filtrate by differential centrifugation as described (Cummings et al. 1979a). Low temperature cytochrome spectra were monitored on the final high speed mitochondrial pellet and were in excellent agreement with previously published spectra. DNA was prepared from mitochondria on DAPI-CsCl gradients exactly as described before. The yield in DNA was usually between 200 and 400 μg.

Preparation of Mitochondria and Mitochondrial RNA
All procedures used for generating mtRNA were identical to those used for producing mtDNA up to the point of the final, high speed, mitochondrial pellet. At that point, mitochondria were suspended in 40 ml of isolation buffer and were loaded onto four blurred-step sucrose gradients. These were one step gradients of 1.9 M sucrose and 0.9 M sucrose in 10 mM Tris, pH 7.6, 100 mM NH4Cl poured 3–4 h before use and stored at 4°C. This allowed the interface between steps to diffuse slightly or "blur". Mitochondria were banded at the interface zone by centrifugation in the SW27 rotor for 60 min at 23,000 rpm with gentle rotation for 60 min followed by 30 min of treatment without rotation. Thirty to forty grams of pelleted mycelia and protoplasts were disrupted in 100 ml of isolation buffer (50 mM KH2PO4, pH 7.6, 0.5 mM EDTA, 0.66 M sucrose) at 4°C. This allowed the interface between steps to diffuse slightly or "blur". Mitochondria were banded at the interface zone and the bulk of contaminating nuclei sedimented through this interface. The mitochondrial band was harvested from the top, diluted in 10 mM Tris, pH 7.6, 100 mM NH4Cl and sedimented in a Sorvall centrifuge at 20,000 rpm for 10 min at 4°C. Gradient purified mitochondria were lysed in 50 mM Tris, pH 7.4, 10 mM EDTA, 0.1 mM aurotricarboxylic acid, 2% SDS. The lysate was extracted once in phenol:chloroform:isoamyl alcohol (24:24:1), and the aqueous phases were re-extracted twice with chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 0.1 volumes of 3M sodium acetate, pH 5.0 and 2.5 volumes of ethanol. Nucleic acids were precipitated at -20°C for 18–20 h. The nucleic acid precipitate was sedimented at 15,000 rpm for 20 min in the Sorvall and the pellet was dissolved in 6M guanidine-HCl, 2.5 M sodium acetate, pH 5.0, 0.1 M β-mercaptoethanol. Three ml of this solution was layered onto a 1 ml cushion of CsCl-EDTA (5.7 M CsCl, 0.1 M EDTA) and centrifuged in the SW 50.1 rotor at 44,000 rpm, 21 h, 18°C (adapted from Flyvbjerg and Schimke 1979). The RNA pellet produced was rinsed once in ice cold deionized water, suspended in 10 mM Tris pH 7.4, 1 mM EDTA, and was immediately precipitated with 0.1 volumes of 3 M sodium acetate, pH 5.0 and 2.5 volumes of ethanol.

Alternatively, RNA was prepared from gradient purified mitochondria by the high salt precipitation method (Battey and Clayton 1978; Nagley and Clayton 1980).

Cloning Mitochondrial DNA
A complete EcoRI restriction digest of native mtDNA was cloned into the EcoRI site of pBR325 (Bolivar 1978) either as the "shot-gun" experiment or as isolated EcoRI fragments.

Restriction Digestion and Electrophoresis Conditions
Restriction endonucleases were purchased from BRL (Bethesda Research Labs), and digestions were conducted as indicated by them. When multiple, complex digests were employed, the following buffer proved very useful: 106 mM Tris, pH 7.4, 6 mM MgCl2, 7 mM dithiothreitol, 100 mM NaCl. Restriction digests were electrophoresed through 0.4–2.0% agarose (BRL) using Tris-acetate buffer (40 mM Tris, pH 7.4, 5 mM sodium acetate, 1 mM EDTA), at room temperature, 90 V., 4–7 h.

RNA was electrophoresed on CH3HgOH-agarose gels according to Bailey and Davidson (1976) or on glyoxal-agarose gels according to McMaster and Carmichael (1977).

Isolation of DNA Fragments from Agarose Gels
Uncloned mtDNA fragments either to be cloned or to be used for restriction analysis were isolated from agarose or acrylamide preparative gels following restriction cleavage, electrophoresis, and ethidium bromide staining. Bands were excised, electroeluted into dialysis tubing at 4°C for 2 h in electrophoresis buffer diluted 20:1. The elution was brought to 0.3 M in sodium acetate pH 5.0, centrifuged in the Sorvall at 12,000 rpm for 60 min, extracted twice with butanol, and precipitated by the addition of 2.5 volumes of ethanol. Cloned EcoRI fragments to be used as nick translated hybridization probes were isolated from two successive preparative agarose gels.

Isolation of RNA from CH3HgOH-Agarose Gels
Ribosomal RNAs to be used as hybridization probes were isolated from 7.5 mM CH3HgOH gels run according to Bailey and Davidson (1976). Adjacent lanes from a gel were processed for staining and photography by washing the gel in 10 mM sodium acetate pH 5.0, 10 mM β-mercaptoethanol for 30 min and then for 30 min in 10 mM sodium acetate pH 5.0. Adjacent lanes were then removed, stained in ethidium bromide, and photographed. They were used for alignment when cutting unstained lanes from the gel. Slices corresponding to unstained rRNAs were cut from the gels, and RNA was eluted from the gel slice according to Crews and Attardi (1980), stored at −20°C as the ethanol precipitate. Bands corresponding to the large and small rRNAs were prepared, and when these were to be used in competition hybridizations, two cycles of purification through CH3HgOH were employed. On analytical CH3HgOH gels, these RNAs showed neither cross contamination nor degradation.

Labeling of DNA and RNA
DNA restriction fragments were labeled by nick translation (Rigby et al. 1977) with the nick translation system from New England Nuclear. The label was α-32P-dCTP.

Ribosomal RNAs were end-labeled essentially by the method of Donis-Keller et al. (1977) with the following modification. Free 5′-OH termini were generated by hydrolyzing RNA at 95°C, 4 min, in 100 mM Tris, pH 9.5, followed by rapid chilling.