Assembly of the Mitochondrial Membrane System

PHYSICAL MAP OF THE OXI3 LOCUS OF YEAST MITOCHONDRIAL DNA

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The oxi3 locus of yeast mitochondrial DNA is currently thought to code for Subunit 1 of cytochrome oxidase (Tzagoloff, A., Macino, G., and Sebald, W. (1979) Annu. Rev. Biochem. 48, 419-441). The respiratory competent strain of Saccharomyces cerevisiae D273-10B/A48 was used to obtain cytoplasmic "petite" clones enriched for genetic markers in the oxi3 locus. The most complex clone studied (DS6) was ascertained to have a mitochondrial genome with a tandemly repeated segment of mtDNA 18.5 kilobases in length. The oxi3 locus was dissected by mutagenesis of DS6 with ethidium bromide and selection of new clones having less complex genotypes. Six derivative clones with genome sizes ranging from 2.3 to 6.1 kilobases have been extensively analyzed. Most of the restriction sites present in the segments of mtDNA retained by the clones have been mapped, thereby providing a detailed restriction map of the oxi3 gene. Based on the physical locations of the most distal oxi3 mutations, the gene spans approximately 10,000 nucleotides and occupies the region of wild type mtDNA from 44 to 58 map units.

A large number of cytoplasmic mutants of Saccharomyces cerevisiae with a deficiency in cytochrome oxidase have been mapped in the region of mitochondrial DNA located between the antibiotic resistance loci oli2 and par (1). These mutations have been collectively assigned to the oxi3 locus. Most mutations in oxi3 are expressed by the absence of Subunit 1 of cytochrome oxidase, one of the mitochondrially synthesized polypeptides of this respiratory complex (1-3). These observations have been interpreted to indicate that Subunit 1 is a gene product of the oxi3 locus (4, 5). This conclusion is substantiated by the recent demonstration that the other two mitochondrial translated subunits of cytochrome oxidase are encoded in separate loci on mitochondrial DNA designated oxi1 and oxi2 (6-8).

Oxi3 mutations scatter over a region of mtDNA considerably in excess of the estimated length of DNA needed to code for a protein of the size of Subunit 1. Morimoto et al. (9) have reported some oxi3 mutants to have deletions of up to 7.5 kb of DNA. Despite the fact that mutations in oxi3 are genetically unlinked, they nonetheless behave as a single complementation group (10). The genetic properties of oxi3 mutants together with recent data on the transcripts issued from the oxi3 region suggest that the gene has a mosaic structure with numerous intervening sequences (11, 12).

In order to determine the organization of the cytochrome oxidase Subunit 1 gene, we have isolated ρ− mutants with different segments of the oxi3 region. The mutants have been characterized genetically and detailed restriction maps have been constructed of their mitochondrial genomes. The restriction maps were used to derive a complete physical map of the oxi3 locus and to assign representative insertions in oxi3 within physically defined limits of mtDNA. The mapping studies indicate the gene to span the region of wild type mtDNA from about 44 to 58 map units. The restriction map of this region has enabled us to sequence the entire gene. These results are presented in the accompanying article (13).

MATERIALS AND METHODS

Yeast Strains and Media—The genotypes of the strains of S. cerevisiae used in this study are described in Table I. The solid media had the following compositions: YPD, 2% glucose, 1% yeast extract, 2% peptone, and 2% agar; YPEG, 2% ethanol, 3% glycerol, 1% yeast extract, 2% peptone, and 2% agar; WO, 2% glucose, Wickerham's minimal salts and cofactors, and 2% agar; P, 3% glycerol, 0.05 M NaKPO4, pH 6.25, 1% yeast extract, 2% peptone, 2% agar, and 2 mg/ml of paromomycin sulfate; C, same as P medium except that 2 mg/ml of chloramphenicol was used instead of paromomycin; R, same as P except for the addition of 2 μg/ml of mutanycin instead of paromomycin.

Isolation of ρ− Clones—The respiratory competent haploid strain of S. cerevisiae D273-10B/A48 was mutagenized with ethidium bromide under nongrowing conditions (19). The culture was spread for single colonies on YPD medium and incubated at 30°C for several days. Small colonies were collected and tested for the retention of mit− markers (oxi1, oxi2, oxi3, cob1, cob2, and pho2) and antibiotic resistance markers (oli2, par, and cap). The details of the procedures used to test for the presence of mitochondrial genetic markers have been described previously (1). This selection yielded the clone DS6 which retained all the oxi3 markers tested but had lost the other mit− and antibiotic resistance loci. The DS6 clone was subjected to a second mutagenesis with ethidium bromide and 2000 new clones were checked for their genotypes. From this second screen, 48 clones were found to have retained different segments of the oxi3 locus. The clones were purified and tested for their genotypic stability. In most cases, they were 80 to 95% stable.

Preparation of mtDNA—The ρ− mutants were grown at 30°C to early stationary phase in liquid YPD medium. Mitochondrial DNA was extracted by the method of Fay et al. (20). Mitochondrial DNA was extracted by lysis with 2% Sarkosyl. The extract was deproteinized with water-saturated phenol and the mtDNA was purified by centrifugation in CsCl gradients (21).
**Physical Map of the Oxi3 Locus of Yeast Mitochondrial DNA**

The deletion mutant M10-150 was a convenient tester for selecting clones with the oxi3 locus. This mutant has been shown to carry a deletion of 7.5 kb spanning the region from 47.5 to 60 map units (9) where most of the oxi3 markers have been mapped. The \( p^c \) clone DS6 was derived from the respiratory competent haploid D273-10B/A48 by mutagenesis with ethidium bromide. The clone was ascertained to have retained all the oxi3 markers represented in our collection of mit\(^-\) mutants including the M10-150 deletion. The loss of genetic markers for the 21 S rRNA, Subunits 2 and 3 of cytochrome oxidase, Subunits 6 and 9 of the ATPase, and apocytochrome \( b \) further indicated that the segment of mtDNA retained in DS6 was confined to the oxi3 region. This was subsequently confirmed by restriction analysis of the DS6 genome.

The oxi3 locus is located between the 14 S rRNA and the ol2 resistance marker (1, 28). Morimoto and Rabinowitz (29) have shown this region to contain sites for Eco RI, Hha I, Pvu II, HinCl I, Hind III, Bgl II, and Bam HI. Digestion of DS6 mtDNA with either Bgl II, Bam HI, Hind III, or Pvu II produced single cuts giving rise to a unit length fragment of 16.5 kb. HinCl I and Hha I each generated two different size fragments, while Eco RI produced four fragments.\(^2\) The positions of these sites on the DS6 mtDNA segment were determined from the estimated sizes of the fragments obtained with single or combinations of different restriction endonucleases. The partial restriction map of the DS6 genome showing only the sites that had previously been mapped on wild type mtDNA is presented in Fig. 1. The restriction map is in good agreement with that reported by Morimoto and Rabinowitz (29) both in terms of the number of different sites and their locations.

The presence in DS6 mtDNA of restriction sites that have been mapped in the mtDNA of D273-10B allows the segment of this clone to be oriented on the wild type map. Furthermore, the absence of some restriction sites in DS6 provides a means of estimating the physical limits (deletion end points) of the DNA (Fig. 1). In these calculations, all the distances have been related to the Pvu II site at 58 map units. Since the mtDNA segment of DS6 is not cleaved by Xho I, the deletion must have been initiated between the Pvu II site at 58 units and a Xho I site that has been mapped at 61 units of the wild type map (29). Based on its unit length of 16.5 kb, the DS6 segment represents the wild type sequence from 35 to 88 units. These measurements are accurate only to within 3 map units.

### Dissection of the oxi3 Region

The presence in the genome of DS6 of all the expected restriction sites indicated it to be a coherent segment of mtDNA without detectable rearrangements or internal deletions. The genotype of DS6 also provided some assurance that the clone contained the entire oxi3 gene. DS6, therefore, appeared to be suitable for the isolation of less complex clones. Our principal aim was to obtain a series of clones with smaller genomes that could be used to determine the detailed restriction map of the oxi3 region and eventually to sequence the Subunit 1 gene.

To further dissect the oxi3 locus, new clones were generated by mutagenesis of DS6 with ethidium bromide. Out of several thousand clones analyzed, 46 had new genotypes. The oxi3 markers retained by some representative clones isolated in this screen are listed in Table II. The following clones were chosen for more detailed analysis: DS6/A400, DS6/A401, DS6/A402, DS6/A407, DS6/A422, and DS6/A462. Each strain was verified to be genetically stable and to have a

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**Table 1**

| Genotypes of wild type and tester strains of S. cerevisiae |
|----------------------------------------------------------|
| Strain | Genotype | Locus | Reference |
|--------|----------|-------|-----------|
| D273-10B/A48 | a, met, \( p^c \), \( \omega^+ \), oxi2 | 14 |
| KL1:4-4A | a, his, trp2, \( p^c \), cap, oxi1, \( \omega^+ \), Cb, OfrP3, par | 15 |
| CB11 | a, adel, \( p^c \), \( \omega^+ \) | R. B. Needleman |
| aM302-34-15C | a, adel, \( p^c \), \( \omega^+ \), pha2 | 16 |
| aM7-40-5B | a,adel, \( p^c \), \( \omega^+ \), cob1 | 17 |
| aM18-12-4A | a, adel, \( p^c \), \( \omega^+ \), cob2 | 17 |
| aM309-4-1B | a, adel, \( p^c \), \( \omega^+ \), oxi1 | 18 |
| aM3-3-6C | a, adel, \( p^c \), \( \omega^+ \), oxi2 | 18 |
| aM3-9-9B | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM5-16-2D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM5-85-1A | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM5-121-5A | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM6-183-1D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM8-227-5D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM9-27-1C | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM10-63-3D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM10-150-4D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM10-237-1C | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM11-125-5B | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM11-224-5D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM12-193-1A | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM13-177-3A | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM15-98-4D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM15-190-2D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM15-233-1C | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |
| aM16-41-4D | a, adel, \( p^c \), \( \omega^+ \), oxi3 | 18 |

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\(^2\) Actually, there are two close Pvu II and several close Eco RI, HinCl I, and Hha I sites. The small fragments generated by these enzymes are not detected on standard agarose gels and were therefore missed in the original mapping studies of Morimoto and Rabinowitz (29).
homogeneous population of mtDNA. The presence of common genetic markers among some of the clones suggested partial overlaps in their genomes. It was also important that collectively the clones were capable of restoring wild type growth to all the oxi3 mutants used in the original screen.

**Physical Map of the DS6 Mitochondrial Genome**—The unit lengths of the mtDNAs retained in the clones derived from DS6 ranged from 2.3 to 6.1 kb. Even though the genomes studied contained numerous restriction sites, it was possible to construct fairly complete maps of the mtDNAs by analyzing the products formed in single and multiple enzyme digests. These analyses were confined to some 14 endonucleases and did not include Alu I and Rsa I which produced too many fragments to permit accurate mapping of the sites.

The physical maps of the mtDNAs were consistent with the genotypes of the clones. For example, the mtDNA segment of the relatively simple clone DS6/A400 has a subset of the genetic markers present in DS6/A401 (Table II). As expected, the segment of DS6/A400 was found to be completely included within the larger genome of DS6/A401. A similar overlap exists between DS6/A407 and DS6/A422. On the other hand, clones such as DS6/A400 and DS6/A402 exhibiting a limited number of common markers have genomes with only partial sequence overlap. With the exception of DS6/A462, the restriction maps of all the clones were completely consistent, suggesting they had retained contiguous segments of the DS6 genome. The mtDNA of DS6/A462, however, has a small internal deletion and inversion of part of its sequence. This type of rearrangement is relatively rare but has been noted previously in a p- clone containing part of the cytochrome b gene (27). Since the genome of DS6/A462 covers the region of oxi3 between the unique Bgl II site at 50 and the Bam HI site at 53 units, the physical map of this span was obtained from DS6/A422 and the parental DS6.

**FIG. 1. Locations of the HincII, Hpa I, Eco RI, Bgl II, Bam HI, HindIII, and Pvu II sites on the DS6 genome.** The DS6 mtDNA has been linearized to indicate the correspondence of restriction sites with the wild type genome of D273-10B (29). All distances have been related to the Pvu II site at 58 map units reported by Morimoto and Rabinowitz (29). The HincII and Xba I sites at 33 and 62 map units, not present in DS6/A401, are also shown. The orientation of DS6 is provided by the par and oli2 markers. The deletion end points of DS6 are not precisely known. The approximate limits are indicated by the dashed parts of the line.

**FIG. 2. Partial restriction maps of the mitochondrial genomes retained in DS6 and the low complexity clones.** The restriction sites on DS6 from 44 to 58 map units are indicated on the outer circle. The physical limits of the other genomes are shown by the arcs within the circle. The dashed line in DS6/A462 indicates the internal deletion in the mtDNA segment of this mutant. The map units are shown in the inner circle. The symbols used for the restriction sites are: ▲, HincII; △, Hpa I; ○, Hae III; ●, Mbo I; □, Mbo II; ●, Taq I; ■, Hph I; ◊, HindIII; ○, Hha I; ○, Eco RI; ■, Bgl II; ○, Bam HI; ●, HindIII; and □, Pvu II.

**TABLE II**

| Genotypes of p- Clones | 10-150 | 8-227 | 15-98 | 15-190 | 12-193 | 3-9 | 10-63 | 5-121 | 11-224 | 5-85 | 15-233 | 16-41 | Unit length of mtDNA |
|------------------------|--------|-------|-------|--------|--------|-----|-------|-------|--------|-----|--------|-------|---------------------|
| Clone                  |        |       |       |        |        |     |       |       |        |     |        |       |         |
| DS6                    | +      | +     | +     | +      | +      | +   | +     | +     | +      | +   | +      | +     | 16.5 kb             |
| DS6/A401               | -      | -     | +     | +      | -      | -   | -     | -     | -      | -   | -      | -     | 6.1 kb              |
| DS6/A402               | -      | -     | -     | -      | -      | -   | -     | -     | -      | -   | -      | -     | 5.2 kb              |
| DS6/A407               | -      | -     | -     | -      | +      | -   | +     | +     | +      | +   | +      | +     | 2.6 kb              |
| DS6/A408               | -      | -     | -     | -      | +      | -   | +     | +     | +      | +   | -      | -     | 3.1 kb              |
| DS6/A409               | -      | -     | -     | -      | -      | -   | -     | -     | -      | -   | -      | -     | 2.3 kb              |
| DS6/A410               | -      | -     | -     | -      | -      | -   | -     | -     | -      | -   | -      | -     | 2.6 kb              |
| DS6/A411               | -      | -     | -     | -      | +      | -   | -     | -     | -      | -   | -      | -     | N.D.**              |
| DS6/A412               | -      | -     | -     | -      | -      | -   | -     | -     | -      | -   | -      | -     | N.D.**              |

**N.D.** not determined.
The results of the restriction mapping on the simpler clones allowed the derivation of the DS6 map from 44 to 58 units (Fig. 2). The overall map was constructed by connecting the genomes of the simpler clones through the common sites in the overlapping regions. As mentioned above, the only ambiguity occurred between the Bgl II and Bam HI sites. This problem was resolved by preparative isolation of the 1.9-kb Bgl II-Bam HI fragment from DS6. The Hint I, Hpa II, Hae III, Mbo I, and Mbo II sites in this fragment were mapped by secondary cleavages of the DNA with different combinations of these enzymes. The composite map shown in Fig. 2 was verified by digestion of the DS6 genome with single enzymes that produce discrete size fragments. In all cases, the sizes of these cleavage products were consistent with the assigned positions of the restriction sites.

**Location of oxi3 Mutations**—The mutations in the oxi3 testers used in the present study have previously been ordered relative to each other by recombinational analysis (1). The restriction maps of \( \rho^+ \) genomes allow them to be assigned to physically defined regions of the oxi3 locus. Based on the deletion end points of the different mtDNA segments, all the mutations are located between 42 and 57.5 units (Fig. 3). These limits, imposed by the extreme edges of the DS6/A401 and DS6/A407 genomes, also provide a measure of the length of the oxi3 gene. From the DNA sequence of the oxi3 region, the Subunit I gene has its NH\(_2\) and COOH termini at 43.7 and 58 units, respectively (13). Due to the relatively large sizes of the \( \rho^+ \) segments, the mutations can only be mapped within spans of several thousand base pairs. Nonetheless, it is of interest that the mutational sites scatter over the entire oxi3 region. These results speak for a complex gene organization with numerous exons and introns, a conclusion borne out by the DNA sequence (13).

The linear order to the oxi3 alleles shown in Fig. 3 is in substantial agreement with the previously reported genetic map of the locus, except for the three mutations M8-227, M15-98, and M15-190. In the earlier studies, these alleles were placed on the right-hand side of the map (1). This error arose from the use in the genetic mapping of the mutant M11-125 which was subsequently shown to have two separate mutations, one at 44 units and the other at 54 units (9). The retention of the M8-227 marker in DS6/A401 and of M15-98 and M15-190 in DS6/A400 unambiguously localizes these alleles between 42 and 46.5 map units. The inability of the simpler \( \rho^- \) clones to restore glycerol growth to M10-150 (Table II) is apparent from the physical map of Fig. 3. As indicated earlier, the M10-150 deletion includes the sequence between 47.5 and 60 map units. Although clones such as DS6/A402, DS6/A407, DS6/A422, and DS6/A401 contain different parts of the missing sequence in M10-150, none except DS6 itself covers the entire deletion.

**DISCUSSION**

At least three genes in yeast mitochondrial DNA are known to contain intervening sequences. They are the 21 S rRNA (30, 31) and the genes coding for apocytochrome b (32, 33) and Subunit I of cytochrome oxidase (11, 12). The 21 S rRNA and apocytochrome b genes have recently been sequenced, and their intron regions have been identified (30-33). Similar information is still lacking for the Subunit I of cytochrome oxidase. This gene is encoded in the oxi3 locus, a region of mtDNA estimated to be at least 10 kb long.

The present study was undertaken with the aim of isolating \( \rho^- \) clones with mitochondrial genomes spanning different parts of the oxi3 gene. In view of the complexity of the gene both with respect to its sequence length and organization, we felt that a physical dissection of the locus was essential for the derivation of the physical map and sequence analysis. The mitochondrial genome of DS6 reported here has been shown to contain the entire locus as evidenced by the retention of all the oxi3 markers. The DS6 mtDNA segment has a unit length of 16.5 kb encompassing the wild type sequence from 35 ± 3 to 58 ± 3 map units. The presence of all the restriction sites previously found in the oxi3 region of the parental D273-10B strain (29) suggested that the mtDNA segment of DS6 represents a contiguous wild type sequence without any gross internal deletions.

Six independent clones obtained from DS6 have been extensively analyzed. Their genome lengths range from 2.3 to 6.1 kb. Although we had initially intended to isolate clones with smaller mtDNA segments, this proved not to be practically feasible. The genotypes of the clones indicated a sufficient degree of genomic overlap to account for the entire oxi3 locus. For example, with the exclusion of the M10-150 deletion, all the markers originally found to be present in DS6 were retained by one or more of the secondary clones. This was also confirmed by restriction mapping, i.e., clones exhibiting common genetic markers had either inclusive or overlapping segments of mtDNA.

The restriction analysis of the \( \rho^- \) mtDNAs has led to a fairly complete restriction map of the DS6 genome from 44 to 58 map units. All the sites for 14 different restriction enzymes have been mapped in this region. The restriction maps of the mtDNA segments retained in the secondary clones have also allowed the oxi3 mutations to be located on the physical map. The mutations fall between 42 and 57.5 units, which is consistent with previous estimates of the physical location of the Subunit I gene (5, 11, 28). The \( \rho^- \) clones described in this article, together with the physical map of the oxi3 locus, have facilitated the sequencing of this interesting region of the yeast mitochondrial genome. The sequence and identification of the coding or exon regions of the Subunit I gene are reported in the companion article (13).

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