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Genetics

Suppressor of yeast mitochondrial ochre mutations that maps in or near the 15S ribosomal RNA gene of mtDNA

(nonsense suppression/DNA sequence/*rho*⁻ cloning)

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ABSTRACT **A polypeptide chain-terminating mutation in the yeast mitochondrial *oxi 1* gene has been shown to be an ochre (TAA) mutation by DNA sequence analysis. Mitochondrially inherited revertants of this mutation include two types: In the first, the ochre codon has been changed to a sense codon by further mutation in the *oxi 1* gene while, in the second, the ochre codon is still present, indicating the occurrence of an extrageneic ochre suppressor mutation. This mitochondrial ochre suppressor, termed *MSU1*, has been "cloned" in *rho*⁻ strains of yeast and tested against other *oxi 1* mutations. Several additional mutations are also suppressible, and those examined so far are also ochre mutations. *MSU1* does not suppress known frameshift or missense mutations at *oxi 1*. Isoelectric focusing of the gene product (cytochrome oxidase subunit II) from a suppressed-mutant strain indicates that suppression does not involve insertion of charged amino acids. Physical mapping of the mtDNA retained in the *MSU1*-carrying *rho*⁻ clones localizes the suppressor mutation to the gene coding the 15S rRNA or a site not more than 300 base pairs from it. No known tRNA genes occur this close to the 15S rRNA gene, and mtDNA from a suppressor-carrying *rho*⁻ does not hybridize detectably to mitochondrial tRNAs. These results suggest that *MSU1* may be an alteration in the 15S rRNA.**

Nonsense mutations and their suppressors have been of great value for the study of both protein-coding structural genes and the genetic systems that express them. Although the mitochondrial genetic system of yeast (*Saccharomyces cerevisiae*) has been intensively studied over the past few years (1, 2), no defined set of nonsense mutations and suppressors for them has been described. Suppressors of yeast mitochondrial mutations have indeed been reported. They arise as a result of either mutations in nuclear genes (3–6) or second-site mutations in mtDNA (4, 5, 7). However, in the previously described cases, the nature of the suppressible mutations was not determined precisely.

We have been engaged in a study of mutants at the *oxi 1* gene in mtDNA (8–10), a simple uninterrupted gene that codes for the polypeptide cytochrome *c* oxidase subunit II (9, 11, 12). To examine suppression in this gene, we have now analyzed the DNA sequence alteration in the mutant *oxi 1-V25*, which has been reported to be a chain-terminating mutation (13) that can be suppressed by several recessive nuclear mutations (A. Putrament and T. Zoladek, personal communication). We report here that *oxi 1-V25* is an ochre (TAA) mutation.

Among the revertants isolated from this mutant, we have identified a strain that carries an extrageneic ochre suppressor mutation in mtDNA, termed *MSU1*. *MSU1* suppresses other *oxi 1* mutations as well, and those that have been examined so far are also ochre mutations. Mapping of the *MSU1* mutation indicates that it is either in or very close to [within 300 base pairs (bp)] the gene coding mitochondrial 15S rRNA.

MATERIALS AND METHODS

Yeast Strains. (See also Table 1.) The *oxi 1-V25* mutant strain AB1-4D/V25 (13) was obtained from A. Putrament. TF2rho⁰ (*a, his4*) was generated by extensive ethidium bromide treatment (14) of strain TF2 (8). The diploid mutant strain V25T was formed by mating AB1-4D/V25 with TF2rho⁰. V25T-R2 and V25T-R5 were spontaneous revertants isolated from V25T. MOS-5 was a haploid segregant isolated by sporulation of V25T-R5. The suppressor-carrying *rho*⁻ strain ROS-31 was isolated from MOS-5 by limited ethidium bromide treatment as described (8), except that cycloheximide was omitted. The wild-type strain used in the experiments of Figs. 2 and 4 was constructed by mating strain 777-3A (*α, ade1, op1*, [rho⁺]) (15) with TF2rho⁰. Additional *oxi 1* mutants referred to in Table 3 were obtained from B. Weiss-Brummer (16), with the exception of M13-249 (9).

Media and Genetic Techniques. YPEG medium contained 1% yeast extract, 2% Bactopeptone, 3% ethanol, and 3% glycerol. Glucose-containing medium (YPD) and genetic techniques were as described (17).

Isolation of mtDNA and Sequence Analysis. The procedures used have been described (8, 9). In the experiment of Fig. 1, the *oxi 1*-carrying 2400-bp *Hpa* II fragment from each strain was digested with *Hin*fI and the resulting fragments were labeled at their 3′ ends. After cleavage with *Pvu* II, the 186-bp fragment labeled at the *Hin*fI end at position −333 (see ref. 9) was isolated and subjected to chemical sequence determination reactions (18).

Labeling of Mitochondrial Proteins, Immunoprecipitation, and Two-Dimensional Electrophoresis. Yeast cells were labeled with $^{35}SO_4^{2-}$ (carrier free) or [^{3}H]leucine (110 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) as described (19). Isolated labeled mitochondria (20) were solubilized in 1% Triton X-100 and subjected to immunoprecipitation (21) with antiserum directed against holocytochrome oxidase (obtained from G. Schatz). The immunoprecipitates were dissolved in "lysis buffer" (22) and subjected to two-dimensional electrophoresis (9, 22, 23).

Isolation, Electrophoresis, Blotting, and Hybridization of mtRNA. Total mitochondrial nucleic acids were prepared from washed wild-type mitochondria (24). Mitochondria were suspended in 0.05 M Tris chloride, pH 7.4/10 mM EDTA/1% $NaDodSO_4$ and digested for 1 hr at room temperature with proteinase K (100 μg/ml). NaCl was then added to 0.15 M and the suspension was extracted with phenol/chloroform/isoamyl alcohol (50:50:1). The RNA (and DNA) was precipitated with

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Abbreviation: bp, base pair(s).

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Table 1. Key strains

Strain	Nuclear genotype	Mitochondrial genotype
AB1-4D/V25	*a, op1, ade1, met3*	*rho*$^+$, *oxi 1-V25*
V25T	*a/a, op1/+, ade1/+, met3/+, his4/+*	*rho*$^+$, *oxi 1-V25*
V25T-R2	*a/a, op1/+, ade1/+, met3/+, his4/+*	*rho*$^+$, *oxi 1*$^{+"}$
V25T-R5	*a/a, op1/+, ade1/+, met3/+, his4/+*	*rho*$^+$, *oxi 1-V25, MSU1*
MOS-5	*a, met3, his4*	*rho*$^+$, *oxi 1-V25, MSU1*
ROS-31	*a, met3, his4*	*rho*$^-$, *MSU1*

ethanol. Samples were heat denatured in 7 M urea and subjected to electrophoresis in a 2% agarose/10 mM NaP_i, pH 7.0 gel. The gel was blotted to diazobenzyloxymethyl paper as described (25). The RNA–paper was hybridized to DNA probes that had been radioactively labeled by nick-translation (26). Hybridization was at 42°C in 50% formamide/0.75 M NaCl/75 mM Na citrate/50 mM NaP_i, pH 5.5/0.2% $NaDodSO_4$/0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone containing sheared calf thymus DNA at 2.0 mg/ml.

RESULTS

***oxi 1-V25* Is an Ochre Mutation.** The position of the *oxi 1-V25* mutation was estimated to lie approximately one-third of the way into the *oxi 1* gene. This estimate was based on the reported M_r of the fragment polypeptide produced by the mutant (11,400; ref. 13) and on the pattern of recombination between *oxi 1-V25* and *rho*$^-$ deletion mutants that have end points within the gene (8, 16) (unpublished results). To examine the nature of this mutation, mtDNA was isolated from the mutant strain V25T (Table 1) and the sequence of the proximal portion of the *oxi 1* gene was determined. Sequence analysis of a 3′ end-labeled fragment of the transcribed strand revealed that a guanosine residue in the wild-type sequence (Fig. 1*a*) had been changed to an adenosine by the *oxi 1-V25* mutation (Fig. 1*b*). This base-pair alteration changed a wild-type CAA (glutamine) codon to a TAA (ochre) codon in the *oxi 1* mRNA of the mutant (Table 2).

Revertants of *oxi 1-V25* Caused by Intrageneic and Extrageneic Mitochondrial Mutations. Among the revertants of a known nonsense mutation, one might hope to find strains carrying a nonsense suppressor. To this end, we isolated several independent revertants from a diploid strain, V25T, carrying the mitochondrial ochre mutation (Table 1). The revertants fell into two classes with respect to growth on medium containing a nonfermentable carbon source, YPEG: Some were similar or identical to wild type while others grew noticeably slower than wild type at 30°C and 37°C and failed to grow significantly at 18°C.

Two revertants from each class were sporulated to examine meiotic segregation of the revertant phenotype; 4:0 segregation would indicate that reversion was due to a second mitochondrial mutation. This analysis was complicated by the presence of the recessive nuclear *petite* mutation *op1* in V25T (Table 1). Thus, if a revertant were the result of a mitochondrial mutation, all four spores would receive functional mitochondria but the ability to grow on YPEG would segregate 2:2 due to the nuclear mutation *op1*. In fact, 2:2 segregation was observed in all tetrads obtained from each of the four revertants examined. To confirm that those haploid progeny that failed to respire nevertheless had functional mitochondria, all spores were crossed to *OP1*$^+$, *rho*^{0} tester strains (containing no mtDNA). All of the resulting diploids grew on YPEG, showing 4:0 segregation of the revertant phenotype in each case. These results, and those presented below, show that the revertants of both classes are due to mutations in mtDNA.

To check whether the revertants were due to alterations at the ochre codon itself, mtDNA was prepared from three of the revertants and the sequence of the affected region of the *oxi 1* gene was determined. One of the revertants that grew similarly to wild type, V25T-R2, was found to have an intrageneic base-pair substitution that changed the TAA codon to an AAA (lysine) codon (Fig. 1*c* and Table 2). However, a slow-growing revertant, V25T-R5, still had the ochre codon in the *oxi 1* gene (Fig. 1*d* and Table 2). [Another slow-growing revertant, V25T-R1, also retained the ochre codon (not shown)]. Thus, the revertant V25T-R5 must have arisen as a result of an extrageneic mutation that suppresses the *oxi 1-V25* mutation. We have termed this suppressor mutation *MSU1*.

"Cloning" of the Suppressor *MSU1* in *rho*$^-$ Yeast Strains. To facilitate mapping of the suppressor *MSU1*, as well as its genetic manipulation, it was desirable to isolate *rho*$^-$ deletion strains (see ref. 1) retaining reiterated copies of *MSU1* but lacking most of the rest of the mitochondrial genome. *rho*$^-$ strains were generated by ethidium bromide treatment of the haploid *rho*$^+$ strain MOS-5 that carries *MSU1* (Table 1). The *rho*$^-$ strains were then crossed to a haploid *oxi 1-V25* strain (AB1-4D/V25;

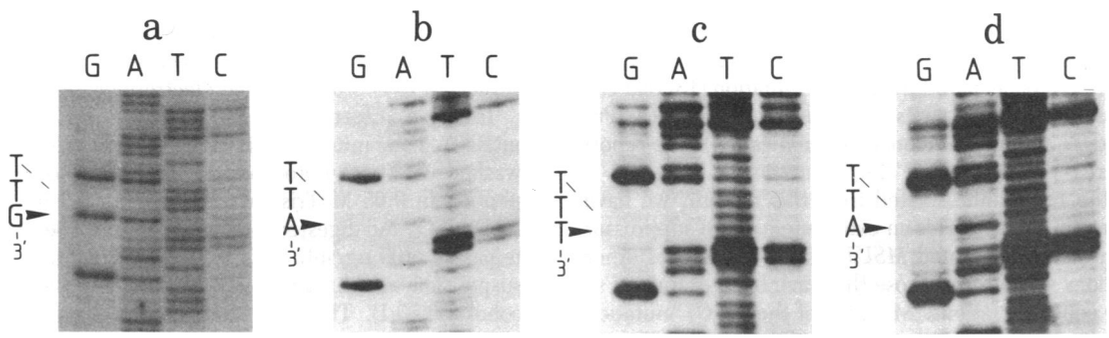

FIG. 1. DNA sequence alterations caused by mutation *oxi 1-V25* and revertants. mtDNA was isolated from wild-type (*a*), mutant V25T (*b*), revertant V25T-R2 (*c*), and revertant V25T-R5 (*d*), and the sequence of a portion of the transcribed strand of the *oxi 1* gene was determined. ► , Bases affected by mutation and reversion; letters, sequence *complementary* to the affected codon.

Table 2. Sequence alterations in the *oxi 1* gene of mutant V25T and its revertants

Strain	DNA	Predicted protein sequence
Wild type	CAT-GGA-*C*AA-ACT-ATT	His-Gly-Gln-Thr-Ile
V25T	CAT-GGA-*T*AA-ACT-ATT	His-Gly-COOH
V25T-R2	CAT-GGA-*A*AA-ACT-ATT	His-Gly-Lys-Thr-Ile
V25T-R5	CAT-GGA-*T*AA-ACT-ATT	His-Gly- ? -Thr-Ile

Base pairs altered in the mutants and revertants are shown in italics and occur at position −180 in the sequence given in ref. 9.

Table 1), and the ability of the resulting diploids to grow on YPEG medium was determined. Those *rho*⁻ strains that retained the ability to suppress the *oxi 1-V25* mutation (≈2% of the total) were isolated and purified by subcloning. Five *rho*⁻ strains that stably retain *MSU1* but not *oxi 1* were obtained.

Other Ochre Mutations Are also Suppressed by *MSU1*. As a first step toward examining the specificity of the suppressor, an *MSU1*-carrying *rho*⁻ strain, ROS-31, was crossed to a collection of 88 *oxi 1* mutants (16). The resulting diploids were then plated on YPEG medium to test the ability of *MSU1* to suppress the mutations. Thirteen mutants were suppressible.

We have identified the DNA sequence alterations in four of these suppressible mutants. All of them have ochre mutations, although the four additional mutants comprise two pairs of strains with identical lesions. Table 3 summarizes these results (ignoring duplicates) and also lists other *oxi 1* mutations of known character (frameshift and missense) that are not suppressed by *MSU1*. We have not yet identified an amber (TAG) mutation in this gene.

As noted above, suppression of *oxi 1-V25* by *MSU1* leads to a cold-sensitive phenotype at the level of cell growth on YPEG medium. However, cold sensitivity does not appear to be due to the suppressor itself, since a strain in which the mutation *oxi 1-M4611* is suppressed by *MSU1* grows slowly but significantly at 18°C.

Suppression of *oxi 1-V25* by *MSU1* Does Not Change the Isoelectric Point of Cytochrome Oxidase Subunit II. The identity of the amino acid(s) inserted during ochre suppression by *MSU1* is at present unknown. We have, however, investigated the possibility that a charged amino acid may be involved by isoelectric focusing of the gene product from the suppressed-mutant strain V25T-R5.

Cytochrome oxidase was purified by immunoprecipitation from cells of V25T-R5 and an isogeneic wild-type strain that had been separately labeled with [^{3}H]leucine and $^{35}SO_4^{2-}$, respectively. The two immunoprecipitates were mixed and subjected together to two-dimensional electrophoresis, after which the distribution of the two isotopes in the isoelectric-focusing dimension was determined for subunit II. Essentially identical distributions were observed for the suppressed-mutant and wild-type proteins (Fig. 2*b*). Since the wild-type amino acid at the position of *oxi 1-V25* (glutamine) is neutral (Table 2), the results indicate insertion of a neutral amino acid (or acids) during ochre suppression by *MSU1*. The isoelectric point of cytochrome oxidase subunit II from the intrageneic revertant V25T-R2, which should have lysine substituted for glutamine (Table 2), was more basic than the wild-type protein, as expected (Fig. 2*a*).

The efficiency of suppression of the *oxi 1-V25* mutation by *MSU1* appears to be 10–20%. This estimate is based on the relative amounts of cytochrome oxidase subunit II, detectable immunologically (27), in extracts of logarithmic-phase cells of V25T-R5, V25T-R2, and wild type (unpublished results).

Physical Mapping of *MSU1* to the 15S rRNA Region of mtDNA. The positions of the known genes on restriction maps of the yeast mitochondrial genome have been determined (1, 28, 29). To determine the approximate location of *MSU1*, mtDNA was prepared from the *rho*⁻ strains described above, radioactively labeled, and hybridized to the mapped restriction fragments produced by *Hin*cII digestion of wild-type mtDNA (not shown). The mtDNAs of all five *MSU1*-carrying *rho*⁻ strains hybridized specifically to the adjacent *Hin*cII fragments 3 and 4. These fragments contain the gene for 15S rRNA, which

Table 3. Suppressibility of *oxi 1* mutations of known character

Mutation	Lesion	Suppressibility	Position
V25	CAA→TAA (ochre)	+	−180
M5611	CAA→TAA (ochre)	+	−318
M4611	TTA→TAA (ochre)	+	40
M13-249	−1 (frameshift)	−	0
M2511	−1 (frameshift)	−	17
M5701	−1 (frameshift)	−	−149
M5631	+1 (frameshift)	−	−149
M5481	CAG→AAG (missense)	−	−339
M5801	GGT→GTT (missense)	−	268

Strains carrying mutations were crossed to ROS-31, and growth of the resulting diploids on YPEG medium at 30°C was scored as suppressibility. Positions of mutations refer to the sequence in ref. 9. The sequence alterations in M13-249 (9) and M5701 and M5631 (10) were determined previously. The other mutations were characterized during the course of this study. (Strains carrying mutations M5701, M5631, and M5801 exhibit a "leaky" phenotype in the absence of a suppressor.)

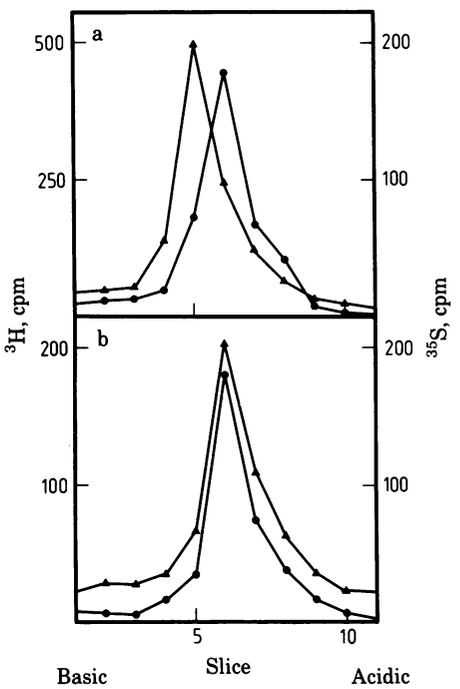

FIG. 2. Isoelectric focusing of cytochrome oxidase subunit II from mixtures of wild-type and *oxi 1-V25*-revertant enzymes separately labeled *in vivo* with different radioisotopes. Cytochrome oxidase was isolated by immunoprecipitation from wild-type yeast that had been labeled with $^{35}SO_4^{2-}$ and from V25T-R2 and V25T-R5 yeast that had been labeled with [^{3}H]leucine. Radioactive mixtures of wild-type and V25T-R2 (*a*) or V25T-R5 (*b*) enzymes were then subjected to two-dimensional electrophoresis. After autoradiography to locate the position of subunit II, strips were cut from the gels and 2-mm slices were cut along the isoelectric-focusing dimension. ^{3}H (▲) and ^{35}S (●) radioactivity in each slice was determined by scintillation counting as described (23).

spans the *Hin*cII site between them, as well as genes coding for several tRNAs (1, 28, 29). No known protein-coding structural genes occur in this region.

To define more precisely the region containing the *MSU1* mutation, we examined the *rho*$^-$ mtDNAs directly by restriction enzyme digestion. First, they were digested with *Bam*HI. Since only a single *Bam*HI site occurs in this region of the mitochondrial genome (28, 29), each *rho*$^-$ mtDNA was expected to yield a single fragment containing the entire mtDNA segment retained and reiterated in the *rho*$^-$ strain. Indeed, *Bam*HI digestion produced a single fragment from each *rho*$^-$ mtDNA (not shown). The fragment lengths were 1950–4100 bp. The *rho*$^-$ strain ROS-31 had retained the shortest sequence, and its mtDNA was examined further.

The relative positions in ROS-31 mtDNA of sites for *Bam*HI, *Hha* I, *Hpa* II, and *Hin*fI were determined by single- and double-digestion experiments. The resulting map (linearized in the region in which the repeated segments join) is shown in Fig. 3, along with the known position of the 15S rRNA sequence relative to these sites (30–32). These results show that the *MSU1*-carrying *rho*$^-$ strain ROS-31 retains most if not all of the mitochondrial 15S rRNA gene and ≈300 bp of additional DNA. [The other four *MSU1*-carrying *rho*$^-$ strains also retained all or most of the region present in ROS-31 (unpublished results).]

The restriction map of ROS-31 mtDNA suggests that the *MSU1* suppressor may be an altered 15S rRNA, since no known tRNA genes map within 1400 bp of the 15S rRNA gene (30). However, the *MSU1* mutation might be in an unknown tRNA gene closer to the 15S rRNA gene and retained in ROS-31. To examine this possibility, radioactively labeled ROS-31 mtDNA was hybridized to total yeast mitochondrial nucleic acids that had been subjected to gel electrophoresis and fixed to diazobenzyloxymethyl paper (Fig. 4, lane b). As expected, the suppressor-carrying DNA probe hybridized strongly to the 15S rRNA and the mtDNA. A weakly hybridizing RNA of higher molecular weight and unknown origin was also detected, but no hybridization was observed in the tRNA position of the gel, even when autoradiograms were greatly overexposed (Fig. 4, lane c). Control experiments with a probe containing a single tRNA gene showed that hybridization to tRNA species is detectable under these conditions (Fig. 4, lane d). These results are consistent with those of the restriction mapping and with the possibility that the *MSU1* suppressor mutation affects the 15S rRNA.

DISCUSSION

We have characterized an ochre mutation (TAA) in the mitochondrial gene *oxi 1* and isolated an ochre suppressor, *MSU1*,

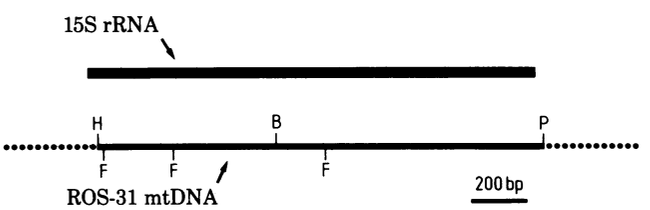

FIG. 3. Restriction map of mtDNA from the *MSU1*-carrying *rho*$^-$ strain ROS-31 showing the region occupied by the 15S rRNA gene. Relative positions of sites for *Hha* I (H), *Bam*HI (B), *Hpa* II (P), and *Hin*fI (F) were determined by single- and double-digestion experiments. The resulting circular map has been linearized in the region in which the reiterated mtDNA pieces are joined (exact location unknown). The sequence retained in ROS-31 could extend as far as the dotted lines on either side but not on both sides together. The map agrees well with other maps of this region (30–33). The location of the 15S rRNA gene is taken from refs. 30–32.

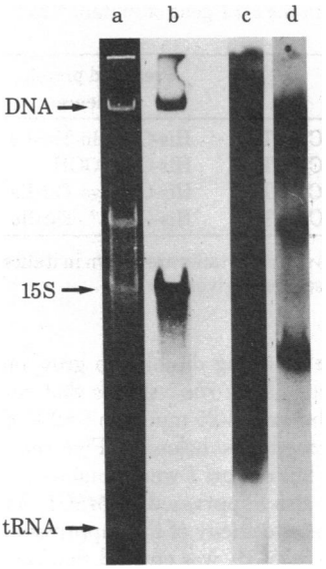

FIG. 4. Hybridization of "cloned" suppressor-carrying DNA to mtRNA (and DNA). Total wild-type mitochondrial nucleic acids were subjected to electrophoresis in an agarose gel (lane a) and blotted to diazobenzyloxymethyl paper. The paper was then hybridized to radioactively labeled mtDNA from *rho*$^-$ strain ROS-31 and autoradiographed (lane b). Also shown are longer exposures of similar gel blots hybridized to ROS-31 mtDNA (lane c) and to a bacterial plasmid carrying a 2900-bp *Mbo* I fragment that contains the gene for mitochondrial valine tRNA and part of the *oxi 2* gene (34) (lane d). The plasmid hybridizes to at least two larger transcripts in addition to the tRNA.

coded in the mitochondrial genome. *MSU1* suppresses not only the ochre mutation used to select it but also several other ochre mutations in the *oxi 1* gene, supporting the notion that it is an informational suppressor. The specificity of *MSU1* as a nonsense suppressor has not been thoroughly studied, since we have not yet identified any amber (TAG) mutations against which to test it. However, *MSU1* does not suppress known frameshift or missense mutations in *oxi 1*, suggesting that it may be specific for ochre (or nonsense) and thus useful for rapid characterization of yeast mitochondrial mutations.

In bacteria and in the nucleo-cytoplasmic genetic system of yeast, nonsense suppression is known to be the result of alterations in tRNA sequences (35, 36) or ribosomal proteins. We have not yet positively identified the molecular species affected by the *MSU1* mutation. However, isolation of *rho*$^-$ clones that carry the *MSU1* suppressor has allowed us to physically map the mutation to the gene coding the 15S rRNA or a site not more than 300 bp from it. No known tRNA gene occurs within 1400 bp of the 15S rRNA gene (30) and, indeed, we cannot detect hybridization between the cloned *MSU1*-carrying DNA and mitochondrial tRNAs. These results suggest that ochre suppression by *MSU1* could be the result of alteration of the small subunit of the mitochondrial ribosome by mutation in the 15S rRNA. However, the possibility that the *MSU1* mutation affects an unknown minor tRNA has not yet been eliminated. Nucleotide sequence studies on the 15S rRNA or the gene coding it will be required to settle this point.

Suppression of nonsense by misreading due to altered ribosome structure is well documented for the bacterial *ram* mutations (37) and the "omnipotent" suppressors of the yeast nucleo-cytoplasmic genetic system (38). In both systems, at least some of the suppressor mutations affect proteins of the small ribosomal subunit (39–41). [One yeast suppressor appears to cause alterations in the large ribosomal subunit (42)]. Alteration of ribosomal proteins is also likely to be the mechanism by which recessive nuclear mutations in yeast suppress the mitochondrial ochre mutation *oxi 1-V25* (A. Putrament and T. Zoladek, personal communication). No nonsense suppressor mutation has yet been described that affects a ribosomal RNA.

In the case of the omnipotent yeast suppressor *SUP46*, suppression of at least one ochre mutation occurs by insertion of serine at the mutant site (43). We do not yet know which amino acid(s) is inserted at mitochondrial ochre codons by the *MSU1* suppressor. Suppression does not appear to involve in-

sertion of charged amino acids, although random insertion of amino acids by *MSU1* would probably be consistent with the results of the isoelectric-focusing experiment (Fig. 2).

The complete sequence of the yeast 15S rRNA gene has recently been reported (31). There are several regions that show homology to *Escherichia coli* 16S rRNA (44, 45), and the sequence can be folded into a predicted secondary structure resembling that proposed for the 16S rRNA of bacteria (46). Thus, *if* the *MSU1* mutation does alter the mitochondrial 15S rRNA, determination of the precise nature of the alteration and examination of the mechanism of ochre suppression should be of interest for study of the function of this molecule in other systems as well. In the case of large ribosomal RNA, mitochondrial mutations conferring resistance to chloramphenicol have been shown to be base-pair changes in a highly conserved region near the 3′ end of the molecule in both yeast (47) and mouse L cells (48).

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