Suppression of a Nuclear \textit{aep2} Mutation in \textit{Saccharomyces cerevisiae} by a Base Substitution in the 5’-Untranslated Region of the Mitochondrial \textit{oli1} Gene Encoding Subunit 9 of ATP Synthase

Timothy P. Ellis, H. Bruce Lukins, Phillip Nagley and Brian E. Corner

Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3168, Australia

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ABSTRACT

Mutations in the nuclear AEP2 gene of \textit{Saccharomyces} generate greatly reduced levels of the mature form of mitochondrial \textit{oli1} mRNA, encoding subunit 9 of mitochondrial ATP synthase. A series of mutants was isolated in which the temperature-sensitive phenotype resulting from the aep2-ts1 mutation was suppressed. Three strains were classified as containing a mitochondrial suppressor: these lost the ability to suppress aep2-ts1 when their mitochondrial genome was replaced with wild-type mitochondrial DNA (mtDNA). Many other isolates were classified as containing dominant nuclear suppressors. The three mitochondrial-encoded suppressors were localized to the \textit{oli1} region of mtDNA using \textit{rho}− genetic mapping techniques coupled with PCR analysis; DNA sequencing revealed, in each case, a T-to-C nucleotide transition in mtDNA 16 nucleotides upstream of the \textit{oli1} reading frame. It is inferred that the suppressing mutation in the 5’-untranslated region of \textit{oli1} mRNA restores subunit 9 biosynthesis by accommodating the modified structure of Aep2p generated by the aep2-ts1 mutation (shown here to cause the substitution of proline for leucine at residue 413 of Aep2p). This mode of mitochondrial suppression is contrasted with that mediated by heteroplasmic rearranged \textit{rho}− mtDNA genomes bypassing the participation of a nuclear gene product in expression of a particular mitochondrial gene. In the present study, direct RNA-protein interactions are likely to form the basis of suppression.

The formation of mitochondrial enzyme complexes responsible for oxidative phosphorylation requires the concerted participation of sets of genes variously located in the nucleus and mitochondrion. The mitochondrial DNA (mtDNA) of the yeast \textit{Saccharomyces cerevisiae} encodes only seven subunits of the various enzyme complexes directly involved in oxidative phosphorylation; the remainder are encoded by nuclear genes. Nuclear genes whose products are required for establishing functional oxidative phosphorylation are designated PET genes. These include nucleus-encoded proteins representing subunits of mitochondrial enzyme complexes involved in energy metabolism, as such. In addition, they include components of mitochondrial machinery involved in organelle biogenesis as a whole, encompassing transcription and translation of mitochondrial mRNA molecules, as well as import of nucleus-encoded proteins into the mitochondrion. Certain classes of PET genes have specific roles in the formation of particular gene products encoded by mtDNA, individually or in limited subsets. The steps in gene expression and protein maturation in which the products of these specialized PET genes are involved include mitochondrial mRNA processing, intron splicing, mRNA stability and translation, as well as post-translational processing and the assembly of multisubunit respiratory enzyme complexes and ATP synthase (for reviews see Dieckmann and Staples 1994; Prescott et al. 1996).

The yeast mitochondrial H^+ -translocating ATP synthase is a multisubunit complex containing at least 11 subunits, 3 of which, subunits 6, 8, and 9, are encoded within the mitochondrial genome (for review see Nagley 1988). The \textit{oli1} gene (also designated \textit{ATP9}) encodes subunit 9, and it is transcribed as part of a polycistronic transcript encompassing the tRNA\textsuperscript{tr} and \textit{var1} genes (Zassenhaus et al. 1984). This multipartite message is processed into its various components, generating the \textit{oli1} mRNA (0.9 kb) encoding subunit 9. The importance of the 5’-untranslated region of the \textit{oli1} mRNA in the biogenesis of subunit 9 was first recognized by the analysis of a temperature-sensitive strain h45 shown to contain a single base insertion 87 nucleotides (nt) upstream of the \textit{oli1} coding region (Ooi et al. 1987).

The formation of the subunit 9 polypeptide also requires nuclear PET genes known as \textit{AEP1} (or \textit{NCA1}) and \textit{AEP2} (or \textit{ATP13}; Ackerman et al. 1991; Finnegan et al. 1991; Payne et al. 1991, 1993; Ziaja et al. 1993). Temperature-conditional mutations in \textit{AEP1} result in
the failure to produce subunit 9, although the mature 0.9-kb oli1 mRNA is readily detectable (Payne et al. 1991). Conditional defects in AEP2 likewise result in the failure to produce detectable levels of subunit 9, but in this case there are no detectable levels of the mature oli1 mRNA. In aep2 mutants, the major precursor mRNA (a 2.1-kb mRNA encompassing both oli1 and tRNA\(^{\text{r}}\)) is detectable at close to, or even higher than, normal levels (Acker man et al. 1991; Payne et al. 1991). These findings suggest a role for Aep2p in the stability of the oli1 message, either directly by virtue of its interaction with oli1 mRNA or through activation of translation of subunit 9 simultaneously preventing degradation of oli1 mRNA.

In this article, we describe the isolation and characterization of three strains carrying mitochondrial mutations that can suppress the temperature-sensitive phenotype resulting from a particular allele of AEP2 (aep2-ts1) (Finnegan et al. 1991). The mitochondrion-encoded temperature-sensitivity-suppressing mutations in all three independently derived strains were mapped to the oli1 region using genetic and molecular techniques. All three suppressing strains were found to contain a T-to-C substitution in mtDNA 16 nt upstream of the oli1 coding region specifying subunit 9. These results strongly suggest that this polynucleotide domain in the oli1 region is critical in the normal functioning of AEP2, probably through interaction of Aep2p with the 5’-untranslated region of oli1 mRNA.

**Materials and Methods**

**Yeast strains, media, and phenotypes:** S. cerevisiae strains and their genotypes are listed in Table 1. Both rho\(^{-}\) (designated with suffix E2) and rho\(^{-}\) strains were constructed using ethidium bromide mutagenesis of the original rho\(^{+}\) strain. The strains S1B, S510, S75, and their derivatives were isolated from a manganese chloride-induced mutagenesis of the aep2-ts1 strain MP3-8C. Strain ORH45 was isolated as an oligomycin-resistant derivative of h45 (see below). D537 was selected among diploids arising from a cross between S510-3 and ORH45, on the basis of its growth on YEPE at 28\(^{\circ}\)C (see results).

Media used were as follows: YEPE [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose]; YEPE [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) ethanol]; synthetic media [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and other supplements depending on the auxotrophic requirements of the strain]; sporulation media [1% (w/v) potassium acetate, 0.1% (w/v) yeast extract, and 0.05% (w/v) glucose]; and Saccharomyces media [1% (w/v) yeast extract, 0.12% (w/v) (NH\(_4\))\(_2\)SO\(_4\), 0.1% (w/v) KH\(_2\)PO\(_4\), 0.01% (w/v) CaCl\(_2\), 0.0005% (w/v) FeCl\(_3\), 0.07% (w/v) MgCl\(_2\), 0.05% (w/v) NaCl, 2% (w/v) ethanol, and other supplements as required]. Solid media contained 1.5% (w/v) agar.

**DNA manipulations:** Total yeast cellular DNA was isolated by a rapid glass-bead vortexing method essentially as described (Hoffman and Winston 1987). Oligonucleotides used in the course of this study are listed in Table 2. PCR amplification was carried out in 100-\(\mu l\) volumes containing 1 \(\mu l\) of yeast whole cell DNA extract, 50 pmol of each of the relevant primers, dATP, dCTP, dGTP, and dTTP (each at 200 \(\mu M\)), and 2.5 units of DYNAzyme (Finzymes, Espoo, Finland) in 1X DYNAzyme PCR reaction buffer [10 mm Tris-HCl pH 8.8, 1.5 mm MgCl\(_2\), 50 mm KCl, 0.1% (w/v) Triton X-100]. Thirty cycles of amplification were performed as follows: denaturation, 1 min (5 min in first cycle) at 95\(^{\circ}\); annealing, 1.5 min at 50\(^{\circ}\); extension, 2.5 min at 72\(^{\circ}\).

**Isolation of aep2 suppressors by manganese chloride-induced mutagenesis:** Screening for suppressors of an aep2 phenotype induced by the preferential mitochondrial mutagen manganese chloride was done essentially as described (Dieckmann et al. 1984). Cells of strains MP3-8C (aep2-ts1) and OL-1(15) (aep2-null) were inoculated into 10 ml YEPE and incubated at 28\(^{\circ}\), with shaking, overnight. Portions (1 ml) of each of these cultures were added to fresh YEPE (10 ml) containing MnCl\(_2\) (0, 2, 3, 4, 5, 6, or 7 mm) and incubated at 28\(^{\circ}\), with shaking, for 24 hr. Each of these cultures was inoculated from an independent starter culture. A total of \(7 \times 10^6\) cells from each of these cultures were plated onto YEPE plates and incubated under nonpermissive conditions to select for suppressors. 36\(^{\circ}\) for MP3-8C and 28\(^{\circ}\) for OL-1(15).

**Ethidium bromide-induced production of rho\(^{-}\) and rho\(^{0}\) strains:** Petite strains retaining incomplete segments of mtDNA (rho\(^{-}\) mutants) were isolated by treating freshly grown cells with ethidium bromide (10 \(\mu g/\) ml) in YEPE (10 ml) for 0, 30, 60, 90, 120, and 390 min at 28\(^{\circ}\). Cells were washed in water and plated onto YEPE plates to obtain single colonies which were then tested for growth on YEPE medium. Cells isolates incapable of growth on ethanol were used for subsequent petite mapping experiments.

For isolation of rho\(^{0}\) strains lacking mtDNA, freshly grown cells were treated with ethidium bromide (25 \(\mu g/\) ml) during 20 generations of propagation in YEPE. Cells were washed in water and plated onto YEPE plates to obtain single colonies. Individual isolates incapable of growth on YEPE were used in subsequent genetic analyses.

**Tetrad dissection of sporulated diploid yeast strains:** Crosses of haploid cells of opposite mating types were performed on a YEPE plate at 28\(^{\circ}\) overnight. Diploids were selected by transferring the mating mixture onto plates containing synthetic media supplemented with the appropriate auxotrophic requirements. Diploids were then plated onto YEPE medium to test for respiratory competence, selecting isolates capable of growth on ethanol. Verified rho\(^{-}\) diploids were transferred to solid sporulation media and grown at 23\(^{\circ}\) for 5–7 days to promote sporulation via nitrogen starvation. Tetrad dissection was performed essentially as described (Johnston and Mortimer 1959). The colonies arising from viable spores were then picked and plated onto the relevant media to test auxotrophic markers and respiratory competence at 23, 28, and 36\(^{\circ}\).

**Mitochondrial translation products:** Mitochondrial translation products were preferentially labeled by incubating cells with \([\text{{[^{35}S]}}]\)-sulfate in the presence of cycloheximide, according to published methods (Murphy et al. 1980). Cells were grown initially at 28\(^{\circ}\), with subsequent radiolabeling carried out at both 23 and 36\(^{\circ}\).

**Isolation of oligomycin-resistant derivative of strain h45:** Cells of strain h45 were grown in 10 ml YEPE overnight, with shaking, at 28\(^{\circ}\). A total of \(5 \times 10^6\) cells were then spread onto YEPE plates supplemented with oligomycin (5 \(\mu g/\) ml) and incubated at 28\(^{\circ}\) for 3–7 days. Colonies were picked and re-streaked onto fresh oligomycin-supplemented plates, to verify oligomycin resistance, as well as YEPE plates incubated at 28 and 36\(^{\circ}\) to verify retention of the temperature-sensitive phenotype characteristic of h45 (inability to grow on ethanol at 36\(^{\circ}\)).

**DNA cycle sequencing of the oli1 region of mtDNA:** Thermal-cycle sequencing of PCR products specific to the oli1 region of mtDNA was performed on whole cell extracts using the Cyclist DNA Sequencing Kit (Stratagene, La Jolla, CA).
TABLE 1

Yeast strains used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Nucleus</th>
<th>Mitochondria</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>J69-1B</td>
<td>MATα adel his6</td>
<td>[rho+]</td>
<td>Macreadie et al. (1983)</td>
</tr>
<tr>
<td>SC167</td>
<td>MATα adel</td>
<td>[rho+]</td>
<td>Payne et al. (1991)</td>
</tr>
<tr>
<td>MP3-8C</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+]</td>
<td>Finnegan et al. (1991)</td>
</tr>
<tr>
<td>MP3-8C(15)</td>
<td>MATα adel [aap2-ts1::LEU2]</td>
<td>[rho+]</td>
<td>P. M. Finnegan</td>
</tr>
<tr>
<td>S1B</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+ sup-m+]</td>
<td>This study</td>
</tr>
<tr>
<td>S510</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+ sup-m+]</td>
<td>This study</td>
</tr>
<tr>
<td>S75</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+ sup-m+]</td>
<td>This study</td>
</tr>
<tr>
<td>S1BE2</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+]</td>
<td>This study</td>
</tr>
<tr>
<td>S510E2</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+]</td>
<td>This study</td>
</tr>
<tr>
<td>S75E2</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho+]</td>
<td>This study</td>
</tr>
<tr>
<td>S510-3</td>
<td>MATα adel [aap2-ts1]</td>
<td>[rho- sup-m+]</td>
<td>This study</td>
</tr>
<tr>
<td>OL-1(15)</td>
<td>MATα his3 [aap2::LEU2]</td>
<td>[rho+]</td>
<td>Finnegan et al. (1991)</td>
</tr>
<tr>
<td>OL-1(15)E2</td>
<td>MATα his3 [aap2::LEU2]</td>
<td>[rho+]</td>
<td>P. M. Finnegan</td>
</tr>
<tr>
<td>h45</td>
<td>MATα adel his6</td>
<td>[mit- (oli1-h45-ts)]</td>
<td>Ooi et al. (1987)</td>
</tr>
<tr>
<td>ORh45</td>
<td>MATα adel his6</td>
<td>[mit- (oli1-h45-ts) oli1-154']</td>
<td>This study</td>
</tr>
<tr>
<td>D537b</td>
<td>MATα/MATα adel/adel</td>
<td>[rho+ sup-m+ oli1-154']</td>
<td>This study</td>
</tr>
</tbody>
</table>

1 sup-m+ denotes the presence of the mitochondrion-encoded suppressor of aep2-ts1.

TABLE 2

Oligonucleotides used for sequencing the oli1 region of mtDNA

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Annealing site</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>oli1#1</td>
<td>cacggagccggaacccccaagag</td>
<td>-357 to -332</td>
<td>Sense</td>
</tr>
<tr>
<td>oli1#2</td>
<td>accaatctgtagatacctgtcc</td>
<td>+31 to +54</td>
<td>Antisense</td>
</tr>
<tr>
<td>oli1#9</td>
<td>tatattcattttggacagc</td>
<td>-4 to +20</td>
<td>Sense</td>
</tr>
<tr>
<td>oli1#4</td>
<td>tatcaccggaatataaagaattgaacc</td>
<td>+201 to +230</td>
<td>Antisense</td>
</tr>
</tbody>
</table>

Numbers indicate nt positions relative to the oli1 reading frame; +1 corresponds to A of the start codon AUG.

1 Sense and antisense oligonucleotides anneal to the noncoding strand and coding strand, respectively.

Nucleotide precursors consisted of appropriate mixtures of all dNTPs and a particular dideoxyNTP, including [33P]-dATP in all samples. Primers are listed in Table 2. Sequencing was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The amplification of sequence was over 25 cycles of the following conditions: denaturation, 30 sec at 96°C; annealing, 15 sec at 50°C; extension, 4 min at 60°C. Results were analyzed on an Applied Biosystems 373A sequencer.

RESULTS

Manganese chloride-induced mutagenesis of strains with defective aep2 alleles: Manganese chloride preferentially mutagenizes mtDNA (Putrament et al. 1973) and was thus chosen for the generation of mtDNA-encoded suppressors of temperature-sensitive and null alleles of aep2. The strains MP3-8C (aep2-ts1) and OL-1(15) (aep2-null) (Table 1) were grown in various concentrations of manganese chloride and plated onto YEPE media to test for suppression of the respective aep2 defects. From a starting inoculum of 7 x 10⁶ cells in each case, treated cells from MP3-8C were incubated at 36°C to identify suppression of the temperature-sensitive defect while cells derived from OL-1(15) were grown at 28°C in an attempt to rescue the absolute defect in this strain. After 2-wk incubation on YEPE there were 158 colonies on YEPE plates incubated at 36°C derived from cells of the treated MP3-8C cultures. In contrast, there were no colonies on YEPE plates among cells plated from the treated OL-1(15) cultures. This suggests that it is very difficult, if not impossible, to isolate suppressors of the
null allele of *aep2*, indicating that there may be a physical requirement for the presence of Aep2p, which cannot be overcome by second site suppression.

**Discrimination between nucleus-encoded and mitochondrial-encoded suppressors:** Suppressors were mapped to either the mitochondrial or the nuclear genome using a series of genetic tests. In the first test, our aim was to separate dominant nuclear suppressor mutations from mitochondrial and recessive nuclear mutations. Of the 158 colonies able to grow on YEPE at 36°C isolated after the mutagenic treatment of MP3-8C, 70 were chosen randomly for further analysis. Each of these 70 isolates was made rho0 (devoid of mtDNA) by ethidium bromide treatment, which removes endogenous mtDNA, one possible site of the suppressing mutation. To test for retention of the suppressing phenotype, each of the 70 rho0 derivatives was crossed to the *aep2*-null strain OL-1(15) and the resultant diploids in each case were tested for growth on YEPE media at both 23 and 36°C. Those isolates that contain a dominant nuclear suppressor of *aep2-ts1* (the only functional *aep2* allele in this cross) generate diploids that can grow on YEPE at 36°C, the nonpermissive temperature. This phenotype accounted for 67 of the 70 suppressor strains. Tetrad analysis was carried out on sets of at least eight tetrads of the diploids resulting from each of three randomly selected representatives of these suppressor strains crossed with OL-1(15). The Leu+ phenotype of a spore was used as a marker for the presence of the disrupted *aep2*-null allele; conversely, a Leu− phenotype was used to infer the presence of the nondisrupted *aep2*-ts1 allele. In spores inferred to carry the *aep2*-ts1 allele, the presence of suppression was assessed by testing for the phenotype representing ability to grow on ethanol at 36°C. All *aep2*-ts1 spores were found to have such a suppressed phenotype, suggesting that an intragenic reversion event at the *aep2* locus (or a closely linked locus) resulted in the suppressed phenotype in these cases. Moreover, if the 3 selected isolates are truly representative of all 67 suppressor strains that, after conversion to rho0 cells, yielded diploids capable of growth on ethanol at 36°C after crossing to OL-1(15), all could be considered intragenic revertants within the *aep2* locus; but this has not been tested in every case.

The three isolates, designated S1B, S510, and S75, that were identified as not carrying dominant nuclear mutations by the preceding test (Figure 1A) and were therefore candidates for carrying mitochondrial or recessive nuclear mutations were subjected to a second genetic test. This test was designed to discriminate between single recessive nuclear mutations and mitochondrial mutations and comprised tetrad analysis of the diploids constructed from these three atypical suppressor strains in the preceding test. Thus, the diploids made by crossing OL-1(15) with each of S1BE2, S510E2, and S75E2 were sporulated at the permissive temperature (23°C), and tetrads were analyzed (Table 3). Leu− spores inferred to be carrying the *aep2*-ts1 allele were tested to see if any of them had the suppressed phenotype (ability to grow on YEPE at 36°C). In this manner, a recessive suppressor unlinked to *AEP2* that was masked by the presence of a dominant wild-type allele in the diploid should be revealed in approximately half the haploid *Leu−* spores. The proportion of *Leu−* spores capable of growth on ethanol at 36°C would be expected to be 100% in the case of a recessive suppression event involving the *aep2*-ts1 allele, or between 50 and 100% for a recessive suppressor linked to *AEP2*. However, no such spores were observed among more than 20 tetrads examined in each case, arguing against the presence of a recessive nuclear suppressor and suggesting that the most likely origin of suppression was mutation(s) in the mitochondrial genome. That the spores did not in general convert spontaneously to the petite rho0 state (involving partial deletion or total loss of mtDNA, respectively) is revealed by the fact that the large majority of *Leu−* spores was capable of growth on YEPE at 36°C; the occasional *Leu−* spore unable to grow on YEPE is presumed to have arisen through mutation to the

![Figure 1](image-url)

**Figure 1.**—Diagnostic matings to determine mode of inheritance of *aep2*-ts1 suppressing mutations. Data illustrate properties of isolates from the parent MP3-8C (*aep2*-ts1) classified as containing a dominant nuclear suppressor (NS1) or a mitochondrial-encoded suppressor (S1B, S510, S75). Growth of cells on YEPE at indicated temperatures is depicted. (All strains grew on YEPD at all temperatures tested.) (A) Phenotypes of diploids from mutant isolates made rho0 and mated to OL-1(15) (*aep2*-null rho0 strain) and corresponding haploid strains. Key to strains: 1, OL-1(15); 2, NS1E2; 3, NS1E2 × OL-1(15); 4, S1BE2; 5, S1BE2 × OL-1(15); 6, S510E2; 7, S510E2 × OL-1(15); 8, S75E2; 9, S75E2 × OL-1(15). (B) Phenotype of diploids of mutant isolates classified as containing a mitochondrion-encoded suppressor mated to OL-1(15)E2 (*aep2*-null rho0 strain) and corresponding haploid strains. Key to strains: 1, OL-1(15)E2; 2, S1B; 3, S1B × OL-1(15)E2; 4, S510; 5, S510 × OL-1(15)E2; 6, S75; 7, S75 × OL-1(15)E2.
petite state. The data obtained (Table 3) thus show no
evidence for a recessive nuclear mutation being wholly
responsible for the suppression of the phenotype re-
sulting from the aep2-ts1 allele; rather the data are con-
sistent with the contention that the suppressor is located
in the mtDNA.

To further explore the latter possibility, the rho+ strains
S1B, S510, and S75 were each mated to OL-1(15)E2, a
rho0 derivative (devoid of mtDNA) of the aep2-null strain
used in the previous sets of matings. The three resultant
diploid strains were able to grow on ethanol at both 23
and 36° (Figure 1B). As the only difference between
this set of matings and the previous set was the source
of mtDNA (in this experiment, from the suppressed
strain in each case) there must be a component within
the mtDNA that contributes to the suppression of the
aep2-ts1-resultant phenotype. This putative determinant
is denoted sup-m. Sets of at least 20 tetrads were analyzed
from each of these matings. The results demonstrated
that all Leu+ spores (indicative of the nondisrupted
allele at the AEP2 locus, aep2-ts1) were suppressed, be-
cause all tetrads showed invariant cosegregation in two
of the spores of the Leu+ phenotype and the ability
to grow on ethanol at 36°. This is the result expected for
a mitochondrial-encoded suppressor of aep2-ts1. Con-}
versely, the remaining two spores displayed both the
Leu+ phenotype (indicative of the disrupted AEP2 loc-
us) and the absolute inability to grow on ethanol. This
result indicates the allele specificity of the suppression
by the putative mitochondrially encoded determinant
sup-m, since none of the spores bearing the null allele
of AEP2 was suppressed, even though all were expected
to have inherited the suppressor-bearing mtDNA. If the
suppression of the aep2-ts1-resultant phenotype was
multigenic, with both mitochondrial and recessive nu-
clear components, then up to half of the Leu+ spores
would be suppressed if the recessive nuclear component
was unlinked to aep2-ts1 (because an independently seg-
regating hypothetical nuclear factor would be involved).
Alternatively, all Leu+ spores would be suppressed if
the recessive suppressor arose from an intragenic muta-
tional event at the AEP2 locus (initially aep2-ts1). While
the evidence presented above points unambiguously to
a mitochondrial component (sup-m) in the suppression
of the aep2-ts1-resultant phenotype, it remains a formal
possibility that there is an intragenic mutation at the
AEP2 locus that remains silent in a background with
wild-type mtDNA, but contributes to suppression in the
presence of a second mutation that is within a modified
mtDNA molecule.

Given that we had apparently isolated three mito-
chondrial suppressors of aep2-ts1, it became important
to determine whether the suppressors were stable or
unstable during growth in nonselective conditions. Sta-
bility of suppression might indicate a homoplasmic
point mutation in mtDNA, whereas unstable mutations
might indicate a heteroplasmic rearrangement of part
of the mitochondrial genome. Such heteroplasmic re-
arrangements provide the molecular basis of suppress-
ion so far described (Dieckmann and Staples 1994;
Grivell 1995) by mitochondrion-encoded suppressors
of mutant alleles at PET loci, where mutations inhibit
expression of particular mitochondrial gene products
at the post-transcriptional level. In these reported cases,
the rearrangements generate heteroplasmic rho− mtDNA
genomes (carrying deletions of some mtDNA sequences)
in which the mitochondrial gene in question is now
flanked by upstream expression-controlling sequences
from a different mitochondrial gene (reviewed in Dieck-
mann and Staples 1994; Grivell 1995). Suppressed
strains of this heteroplasmic type (which carry both nor-
mal and rearranged mtDNA molecules) are characteris-
tically unstable in prolonged vegetative growth.

The stability of the mitochondrial genome of each of
the three suppressor strains, S1B, S510, and S75, was
tested by putting these strains through multiple passages
in glucose-containing media (at least 25 generations),
after which cells were plated onto YEPD media. The
resultant colonies were then replicated onto YEPE, incu-
bated at 36°, and tested for retention of the ability to
grow on ethanol at 36°. An initial heteroplasmic re-
arrangement is anticipated to result in a high propor-

<table>
<thead>
<tr>
<th>Segregation patterna</th>
<th>Mating</th>
<th>No. of tetrads analyzed</th>
<th>2 Leu+ EtOH (36)−</th>
<th>1 Leu+ EtOH (36)−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1BE2 × OL-1(15)</td>
<td>21</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>S510E2 × OL-1(15)</td>
<td>24</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>S75E2 × OL-1(15)</td>
<td>20</td>
<td>19</td>
<td>1</td>
</tr>
</tbody>
</table>

a EtOH (36)− denotes spores that grow on YEPE at 23° but not at 36°. EtOH− denotes spores unable to grow
on YEPE at either 23 or 36°.
tion of colonies being either respiratory incompetent (those that retained the rearranged mtDNA molecule homoplasmically, but not full-length mtDNA) or temperature sensitive (those that retained the wild-type mtDNA molecule but were no longer suppressed, having lost the rearranged rho\textsuperscript{−} mtDNA component). The results showed that over 98% of the colonies retained the ability to grow on ethanol at 36°C (data not shown in detail). The stable retention of the suppressing phenotype suggests that the suppression of the aep2-ts1 resultant phenotype is the result of a discrete mutation in the mtDNA genome (that is, the presence of a sup-m allele at a particular locus), but is not due to gross heteroplasmic rearrangements of mtDNA.

Mitochondrial translation products of strains containing suppressing mutations of the aep2-ts1 resultant phenotype: The mitochondrion-encoded suppressor strains grow at similar rates to wild-type strains at 36°C on ethanol-containing media (data not shown). To determine whether this efficient growth rate was due to complete suppression of the aep2-ts1 allele-resultant phenotype at the molecular level, the levels of subunit 9 produced in one of the suppressor strains were determined. Mitochondrial translation products were analyzed from the parent strain MP3-8C (aep2-ts1 strain) and from the putative mitochondrial suppressor strain S1B, after labeling of cells with [35S]sulfate at either 23°C or 36°C (Figure 2). The levels of subunit 9 at 23°C are similar in both the temperature-sensitive and suppressed strains (compare lanes 2 and 4). At 36°C the temperature-sensitive strain MP3-8C (nonsuppressed) had no detectable subunit 9 (lane 5) but the suppressed strain had the highest amount of subunit 9 (lane 3) comparable to that of the control aep2-ts1 strain MP3-8C at 23°C (lane 4). Such high levels of subunit 9 in the suppressed strain at 36°C suggest a strong, stable suppression of the phenotype resulting from the aep2-ts1 allele.

Genetic analysis of rho\textsuperscript{−} petites retaining the aep2-ts1 suppressing mutations in mtDNA: In order to map the mitochondrial suppressor mutations to a locus on the mitochondrial genome, rho\textsuperscript{−} derivatives of the suppressor were generated, in which different portions of the mitochondrial genome are more or less randomly deleted. A diagnostic cross was then carried out to see if each such rho\textsuperscript{−} derivative had retained (sup-m\textsuperscript{+}) or lost (sup-m\textsuperscript{−}) the information required for suppressor activity. Finally the regions of the mtDNA genome retained in the rho\textsuperscript{−} sup-m\textsuperscript{+} isolates were determined using combinations of genetic and molecular techniques.

A series of petite derivatives (deleted for part or all of the mtDNA genome) was isolated from the three mutant strains containing a mitochondrion-encoded suppressor of the aep2-ts1 resultant phenotype, S1B, S510, and S75. Ethidium bromide mutagenesis was applied under mild conditions optimal for generating rho\textsuperscript{−} petites retaining incomplete segments of mtDNA (although some cells may have become rho\textsuperscript{+}, totally lacking mtDNA). To test for retention of the suppressing mutation, the petite isolates from the mutagenesis of each of the three strains were mated to the aep2-null strain OL-1(15) and the resultant diploids were tested for growth on YEPE at 36°C. Those diploids capable of growth on ethanol at 36°C are most likely to contain a mitochondrial genotype generated by a recombination event between the (normal) mtDNA genome of the rho\textsuperscript{−} aep2-null strain and a fragmentary rho\textsuperscript{−} mtDNA genome that retains the suppressing mutation (sup-m\textsuperscript{+}). Such recombined mtDNA molecules would engender suppression of the phenotype resulting from the aep2-ts1 allele in the diploids. Of 60 YEPE\textsuperscript{−} isolates from the ethidium bromide mutagenesis of each of S1B, S510, and S75, about 10% of petite derivatives retained the suppressing mutation (specifically 6, 7, and 6, respectively). Figure 3 shows the phenotype of three of these rho\textsuperscript{−} sup-m\textsuperscript{+} strains, one from each of the three suppressor strains, and the resultant diploids when mated to OL-1(15). The remaining 90% of petite derivatives in each case lack the putative sup-m locus, either by partial deletion of mtDNA (rho\textsuperscript{−} sup-m\textsuperscript{−}) or by complete loss of mtDNA (rho\textsuperscript{0}).

Close linkage of aep2-ts1 suppressors to the oliI locus in mitochondrial DNA: A PCR approach was utilized to determine whether there is a mtDNA segment retained
in common between the rho⁻ strains. The simplifying assumption was made at the outset that the site of mutation is near the oli1 gene, although it was recognized that a mutation in another part of the genome may be causing the resultant phenotypic changes. Therefore, PCR was performed on whole cell DNA from each of the rho⁺ strains with a series of primers (Table 2) specific to the oli1 region (Figure 4A). The results of the PCR are shown in Figure 4B. Use of the primer pair oli1#3 and oli1#4 showed that all of the rho⁻ isolates from each of the three strains retained sequences within the oli1 reading frame because in each case a product of 234 bp was observed (left-hand panels of Figure 4B). Use of primer pair oli1#1 and oli1#2 enabled the rho⁺ clones to be classified as to their retention of mtDNA upstream of the oli1 reading frame. Thus only a subset of petite clones retained sequences that were amplifiable with primer oli1#1 that anneals 357 nucleotides upstream of the oli1 reading frame, generating a 447-bp product with the opposed primer oli1#2 (right-hand panels of Figure 4B; namely, clone 1 of S1B; clones 1, 3, 4, and 5 of S510; and clone 5 of S75). As positive controls for PCR, DNA from each of the parent strains of these petite clones, S1B, S510, and S75, as well as the aep2-ts1 strain MP3-8C was found to generate the relevant PCR product using both sets of primers (lanes S and ts, respectively, in Figure 4B). The critical mapping result in these PCR studies derives from the lack of amplification of a PCR product using primers oli1#1 and oli1#2 and DNA from the remainder of the rho⁺ sup⁻⁺ petites. The upstream limit to the possible site of the sup⁻ allele is formally set at nt 357, because each of these clones retains the suppressing oli1 reading frame (data from primers oli1#3 and oli1#4 in Figure 4B), but does not retain the oli1#1 primer annealing site at nt 357 to 332 relative to the oli1 start codon.

As all the rho⁻ derivatives retain some sequences downstream of the oli1 reading frame, a recombinational mapping approach was used to determine the downstream limit for the possible sites of the sup⁻ allele.
This oligomycin-resistant h45 strain was clear intragenic reversion event at the olig1-154 region of the D537 mtDNA (using primer oli1#3) analysis therefore revealed the clean 2:2 segregation of aep2-ts1 structure mapping in the olig1 region. To provide two reference genetic markers in fine represents the downstream limit (Figure 5). In genetic 2 allele in strain ORh45 (see text). Dotted lines repre- the position of the h45 mutation at nt -87 and the base substitution (-16) that is the candidate for sup-m mutation are also indicated.

Figure 5.—Genetic map of upstream oli1 region of mtDNA (not to scale). Numbers represent nt positions relative to oli1 reading frame (box), +1 corresponding to A of start codon AUG. L and R, respectively, indicate leftmost (upstream) and rightmost (downstream) limits of genetic map position of sup-m corresponding, respectively, to 5' end of primer oli1#1 (Figure 4) and nt position +68 of mutated base generating oli1-154 allele in strain ORh45 (see text). Dotted lines represent mtDNA lying beyond this defined genetic interval. Positions of the h45 mutation (-87) and the base substitution (-16) that is the candidate for sup-m mutation are also indicated.

For mapping purposes, the sup-m allele from a suitable petite clone was incorporated by recombination back into a rho+ mtDNA genome, as follows. ORh45 was mated to the strain S510-3 (rho- sup-m+; petite clone 3 derived from S510) and diploids plated onto YEPE media containing oligomycin, incubated at 36°C. A particular oligomycin-resistant diploid able to grow on ethanol at 36°C (in this case arising from replacement of the h45 allele with the corresponding wild-type allele from mtDNA of S510-3) was selected at random and designated D537. The results of tetrads dissection following sporulation of D537 showed that all spores contained both oligomycin resistance and the ability to grow on ethanol at 36°C (4:0 segregation) in eight out of eight tetrads (data not shown). This demonstrates that the spores containing the aep2-ts1 allele are suppressed, thereby suggesting that the suppressing sup-m mutation in the partially deleted mtDNA of rho- S510-3 has indeed been incorporated by recombination into the full-length rho+ mtDNA genome of D537. Sequencing of the oli1 region of the D537 mtDNA (using primer oli1#3) verified the presence of the G-to-C change at nt 68 within the oli1 reading frame, confirming that the oligomycin-resistant phenotype of D537 was due to the oli1-154 allele derived from ORh45. Furthermore, sequencing toward the upstream region using primer oli1#2 showed the h45 mutation to be absent from the mtDNA of D537. Moreover, a novel base substitution (T to C at nt -16) was observed in D537 mtDNA, and this is a candidate mutation for sup-m (see Figure 5 for a map of the oli1 region and text below for further DNA sequence details).

On the basis that mtDNA of D537 does indeed contain the sup-m suppressor (see next paragraph) and noting that all spore clones from this diploid are oligomycin-resistant, it is possible to conclude that the suppressing mutation in S510 lies between nt -357 (the formal upstream limit from PCR analysis) and nt +68 (the second base pair of the codon affected by the oli1-154 oligomycin resistance allele) of the oli1 region, which represents the downstream limit (Figure 5). In genetic terms, the recombinational events involving mtDNA molecules that generated the rho+ mitochondrial genome of D537 from the rho- mtDNA genome of ORh45 and the rho- mtDNA of S510-3 took place as follows. The downstream event took place somewhere between the position of the h45 mutation at nt -87 and the position of the oli1-154 allele at nt +68. Upstream, the recombination event took place somewhere between nt -357 (the upstream limit of sup-m) and nt -87 (the position of the h45 mutation).

Before drawing strong conclusions from the above DNA sequencing data it was important to verify formally that the suppression of aep2-ts1 in the spores resulting from meiosis of D537 was not due to a subsequent nuclear intragenic reversion event at the AEP2 locus of the type described earlier in this study (see above) rather than the putative sup-m mutation. Therefore, one of the tetrads from D537 was selected and the spores were made rho0 by ethidium bromide mutagenesis to eliminate mtDNA derived from D537. Each such rho0 spore clone derivative was subsequently mated to either of the aep2-null strains MP3-8C(15) or OL-1(15), depending on mating type. The ability of the resultant diploid clones to grow on ethanol at 36°C was tested. Two of the diploid clones showed a temperature-sensitive growth pattern on ethanol (unable to grow at 36°C). Their temperature sensitivity verifies that the only source of suppression of the aep2-ts1 allele in D537 (from the S510-3 haploid parent of D537) was determined by the mtDNA of the D537 spores [such mtDNA is not present in the rho0 × OL-1(15) diploids or the rho0 × MP3-8C diploids]. The diploids arising from the other two spores were unable to grow on ethanol at 36°C, indicating that these spores contain wild-type AEP2 alleles originating from ORh45 (the other haploid parent of D537). This tetrad analysis therefore revealed the clean 2:2 segregation of...
the two alleles of AEP2 represented in the meiosis of D537, namely, aep2-ts1 and wild type.

**Determination of the DNA sequence change in the oli1 region of mtDNA corresponding to aep2-ts1 suppressors:** The sequence of the oli1 region of the aep2-ts1 strain MP3-8C and the three derived strains S1B, S510, and S75 (each containing a mitochondrion-encoded suppressor) between nt -332 and +69 was determined on DNA amplified by PCR using the primers oli1#1 and oli1#4. The primers used for the cycle sequencing were oli1#2 or oli1#3 (see Table 2). The published oli1 sequence (Ooi and Nagley 1986) from the rho- strain 23-3 was used as a reference.

The sequence of the oli1 region in the three suppressed strains and the unsuppressed parent MP3-8C was identical to that of 23-3 within the oli1 coding region, as far as the sequence could be read. This sequence similarity encompassed nt +68 that is the 3' limit of the possible site of the sup-m mutation (see above). Upstream of the oli1 coding region there were found in these strains several differences from that of strain 23-3, conserved among each of MP3-8C, S1B, S510, and S75 (data not shown; see also legend to Figure 4). There was, however, one systematic sequence difference between the temperature-sensitive parent and each of the three independently isolated suppressed strains, namely the T-to-C transition at nt -16 relative to the oli1 start codon. The diploid D537 also contains this mutation (see above), but lacks the h45 mutation. The latter mutation was also not found in MP3-8C, S1B, S510, or S75. To ascertain whether the T-to-C mutation at nt -16 (defining sup-m) was an artifact of the manganese chloride mutagenesis, the oli1 region of mtDNA from each of the six of the suppressed strains characterized as containing dominant nuclear mutations was sequenced. Each of these six strains had a T (not a C) at nt -16. Note further that S1B, S510, and S75 arose within separate incubations, each in a different concentration of manganese chloride, and are thus completely independent. The conclusion is therefore reinforced that the T-to-C transition is indeed the sup-m mutation that determines mitochondrion-encoded suppression of the temperature-sensitive phenotype of strains carrying the aep2-ts1 allele.

**Molecular determination of the aep2-ts1 mutation:** Using a number of primers specific to the AEP2 region (details available on request) the sequence of this gene was determined in MP3-8C (aep2-ts1) and SC167, its parent carrying the wild-type AEP2 gene. Using automated dye terminator sequencing it was found that the only sequence difference between the two sequences in the AEP2 reading frame and flanking regions was a transition at nt 1238 of the AEP2 reading frame (T in SC167 and C in MP3-8C). This sequence difference was confirmed by sequencing both coding and noncoding strands (data not shown). The mutation results in a single amino acid substitution at residue 413 of Aep2p, in which leucine is replaced by proline. Secondary structure prediction using the SOPM algorithm (Geurjón and Delage 1994) suggests that leucine-to-proline change shortens a helical structure by four amino acids.

**DISCUSSION**

This study provides a further important genetic link between the nuclear AEP2 gene and the mitochondrial oli1 gene. Genetic interactions involving these genes were previously inferred (Payne et al. 1991) from a severely impaired accumulation of subunit 9 of ATP synthase in temperature-sensitive aep2 mutants grown at the restrictive temperature. In the current study, the characterization of a base substitution T to C at nt -16 in the oli1 region of mtDNA as a suppressor of the nuclear aep2-ts1 allele enables the 5'-untranslated region of oli1 mRNA to be identified as a likely candidate for a site of molecular interaction (see below), reflecting the genetic interactions between AEP2 and oli1. Our results have led to the characterization of a mitochondrion-encoded suppressor that does not involve complete bypass of a requirement for the nuclear gene product for translation of a particular mitochondrial mRNA, as is the case with heteroplasmic suppression whereby the specific mitochondrial gene expression has been effectively transferred to the direct control of another functional set of PET genes (Dieckmann and Staples 1994; Grivell 1995).

We isolated three primary strains in which mitochondrion-encoded suppression of the temperature-sensitive phenotype arising from the aep2-ts1 allele occurred. Each suppressed strain we characterized as containing sup-m (S1B, S510, and S75) has a stable phenotype, as do all of the 158 suppressors isolated. This phenotype is inconsistent with an unstable heteroplasmic population of mtDNA molecules. The lack of any suppressors that were of the heteroplasmic petite type suggests that the site of putative Aep2p interaction with the oli1 mRNA may encompass not only the 5'-untranslated region of oli1 mRNA but also sequences within the oli1 reading frame itself; thus recombinational events in mtDNA that remove DNA specifying the Aep2p-binding site from the oli1 5'-untranslated region will also perturb the oli1 reading frame to such an extent that functional subunit 9 is no longer produced. A similar explanation has been proposed for the failure to isolate heteroplasmic suppressors of Ms51p in its interaction with COX1 (Decoster et al. 1990). Alternatively, it may be that the recombinational event to produce a heteroplasmic suppressor is difficult to obtain in the 16 nucleotides available between the oli1 start codon and position -16 nt. This could be tested by constructing in vitro a gene fusion with Aep2p detected by a heterologous promoter and its introduction into a suitable aep2 mutant host strain by transformation using microprojectile bombardment.

Another possible explanation for the lack of hetero-
plasmic suppression is that Aep2p has functional interactions relevant to the expression of gene products over and above that of the oli1 gene. If this were so, while it may be possible to bypass expression blocks involving one gene product (for example, subunit 9 of ATP synthase), this cannot readily be achieved simultaneously for other such mitochondrial gene products. Alternatively, there may be multiple functional domains in Aep2p. In this case, while it may be possible to overcome by genetic suppression a defect in one of these domains, as evidenced by the suppression of the temperature sensitivity-conferring allele of AEP2 (aep2-ts1), the loss of several functions cannot be compensated for. Such a scenario is proposed for Cbp6p (Dieckmann and Staples 1994), which has been suggested to be required for not only expression of cytochrome b in mitochondria, but also for maturation of succinate dehydrogenase activity. A less radical kind of multifunctionality of domains in Aep2p would occur if this protein interacts with more than one factor, as would be the case if it is part of a multisubunit complex bound to oli1 mRNA.

The location of sup-m close to the start codon of oli1 suggests that Aep2p has a direct role in the translation of the oli1 message. Such a role for Aep2p may be direct in activating the initiation complex for translation, or Aep2p may be involved in the selection of the initiation codon. In either case, the previously inferred role of Aep2p in the stability/maturation of the oli1 message (Ackerman et al. 1991; Payne et al. 1991) would be indirect, a consequence of impaired translation of the oli1 mRNA. The translation-dependent stability of mRNA has been demonstrated previously in bacteria and yeast (Steel et al. 1996; Linz et al. 1997). Whether there is a role for Aep2p in the maturation of the oli1 message remains to be determined. The proximity of the suppressing mutation to the middle of the mature 0.9-kb oli1 mRNA, far removed from the processing sites, may argue against such a role for Aep2p.

It is likely that the site of the sup-m mutation in the oli1 mRNA is found at a position involved in physical associations with a particular RNA-binding protein. We suggest that these RNA-protein interactions may involve Aep2p directly, such that the restoration of subunit 9 biosynthesis is achieved by the sup-m mutation altering the structure of oli1 mRNA to accommodate the modified structure of Aep2p generated by the aep2-ts1 mutation. The possibility of more complex interactions, involving a number of proteins binding to the oli1 mRNA in order to facilitate translation of subunit 9, remains to be investigated.

It should also be recognized that Aep2p might be functioning in a membrane environment. Indeed, it has been shown that Cbs1p, Cbs2p (Michaelis et al. 1991), Sco1p (Buchwald et al. 1991), Pet54p, Pet111p, and Pet494p (McMullin and Fox 1993), all nucleus-encoded proteins involved in activating specific mitochondrial gene expression, are all membrane associated.

The results described in this article detail a novel type of suppression of a defect in a nucleus-encoded gene product by a single nucleotide change in its mitochondrial target nucleic acid sequence. A related type of suppression, but of opposite polarity, has been observed in the case of four mitochondrial genes (including oli1 itself), which is informative for the possible mechanism of interaction between Aep2p and the 5'-untranslated region of oli1 mRNA.

Mulero and Fox (1993) generated mutations in the 5'-untranslated region of cox2 mRNA by microprojectile bombardment, which prevented biosynthesis of cytochrome c oxidase but did not affect accumulation of cox2 mRNA. Such cox2 mutations could be suppressed by mutations in the PET111 gene. The putative RNA-protein interactions involving Pet111p and the 5'-untranslated region of cox2 mRNA were explored by further mutagenesis of the cox2 region of mtDNA (Dunstan et al. 1997). In a similar manner, Costanzo and Fox (1993) introduced mutations into the 5'-untranslated region of cox3 mRNA, generating a cold-sensitive phenotype for biosynthesis of cytochrome c oxidase, which could be suppressed by a mutation in the nuclear PET122 gene. Further studies revealed additional mutations affecting the structure of Pet122p that could suppress other mutations in the 5'-untranslated region of cox3 mRNA (Weisenberger et al. 1995) and also demonstrated the interactive role of Pet122p, Pet54p, and Pet494p in activating translation of cytochrome c oxidase subunit III encoded by the mitochondrial cox3 gene (Brown et al. 1994; Costanzo and Fox 1995). These interactions are suggested to occur among the 5'-untranslated region of the relevant mitochondrial mRNA, the nucleus-encoded translational activators, and the mitochondrial ribosome at the surface of the inner membrane of mitochondria (Brown et al. 1994; Sanchirico et al. 1998).

Chen and Dieckmann (1997) described a mutation, introduced by microprojectile bombardment into the 5'-untranslated region of cob mRNA, which was suppressed by a consequent mutation in the nuclear CBP1 gene. The analogous situation to those above has also been observed involving the oli1 region and the AEP2 gene (L. Helfenbaum, B. E. Corner, H. B. Lukins and P. Nagley, unpublished data). Nucleus-encoded suppressors of the h45 mutation (lying in the 5'-untranslated region of oli1 mRNA) were characterized within the AEP2 region of chromosomal DNA.

Each of the above-mentioned cases, in which a nucleus-encoded protein (or group of such proteins) is thought to interact with the 5'-untranslated region of a specific mitochondrial mRNA, provides a very useful system for gaining new insights into nucleus-mitochondrion interactions required for mitochondrial function.
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